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**COPPER AND PEROXIDES IN
RADIOBIOLOGY AND MEDICINE**



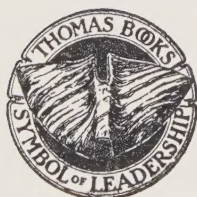
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COPPER AND PEROXIDES IN RADIOBIOLOGY AND MEDICINE

By

JACK SCHUBERT, Ph.D., 1917-

*Associate Technical Director
Nuclear Science & Engineering Corporation
Pittsburgh, Pennsylvania*



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FOREWORD

OF ALL THE essential trace metals, the unique and widespread role of copper has become recognized. The purpose of this book is to explore and review the functions of copper in living organisms and to point out new areas of research. Especial emphasis is given to the involvement of copper in radiobiology. A study of the chelating properties of the drugs employed most successfully to counteract the lethal effects of ionizing radiation revealed that copper is uniquely involved in the mechanisms by which ionizing radiation affects living organisms. This opens the way to new experimental approaches for chemically counteracting radiation injury.

A critical analysis of the different chemical behavior of the two principal oxidation states of copper has led to new interpretations of its biochemical and medical behavior. Equally important has been the stimulus to the design of experiments which otherwise would not have been performed.

Among the topics related to radioprotection and medicine treated in this book are: (1) New approaches to the nature of and the treatment of Wilson's disease; (2) Elucidation of the copper catalyzed oxidations of ascorbic acid and the demonstration of enhanced destruction of ascorbic acid by small concentrations of a chelating agent; (3) New approaches to the role of copper in antipyresis and in the ageing processes; (4) Demonstration of a mechanism of action of small doses of ionizing radiation on a copper protein leading, among other things, to the elucidation of the protective action of hibernation; (5) Simple correlations between molecular structure and protective or sensitizing action of sulfhydryl and other compounds in radioprotection; (6) Explanation of the fact that the lethal effects of ionizing radiation on living organisms vary from a few hundred roent-

gens to hundreds of thousands of roentgens; (7) Inter-dependence and variation of the degree of radiation protection by cyanide and related cuprous chelating agents on both the dose of the agent and the radiation dose.

The book has been written with the needs of the chemist, biologist, and medical investigator in mind. I have attempted to present the chemical and biological aspects with sufficient background information so that these would be comprehensible and useful to both the chemist with little background in biological matters or radiation chemistry, and to the biologist with a limited background in chemistry. It is hoped that the book will serve to stimulate and excite the curiosity of the advanced student and those engaged in active research in radiobiology, medicine, and trace metal biochemistry.

I am highly appreciative for the information and advice given me by Prof. I. M. Klotz of the Department of Chemistry, Northwestern University, on many of the chemical aspects of copper chemistry; and Dr. E. J. Hart of the Chemistry Division, Argonne National Laboratory, who gave me help on many aspects of radiation chemistry. I am also grateful for helpful discussions I have had with former colleagues at the University of Buenos Aires in the Facultad de Ciencias Exactas y Naturales. Naturally, errors of fact, fancy, or concept are mine alone.

I am indebted to my former associates at the Argonne National Laboratory, Mrs. Joan Fried Markley, Dr. Marcia W. Rosenthal, Dr. Arthur Lindenbaum, and especially Mr. William M. Westfall for their help and advice.

JACK SCHUBERT

Pittsburgh

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**COPPER AND PEROXIDES IN
RADIOBIOLOGY AND MEDICINE**

Chapter I

INTRODUCTION

IT IS AN intriguing and curious fact that a large variety of chemical agents which modify radiobiological actions of ionizing radiation can react selectively in the physiological milieu with copper, and only copper in its distinctive oxidation states. Thus, chemical protective agents such as the mercaptans, dithiols, dithiocarbamates, cyanides, azides, and nitriles (E6, P5) complex or chelate preferentially with *cuprous* copper (M8, P10, S33) while chemical sensitizing agents (in mammals) such as the α -alkyl alanines (L3) and quinoids (M25) favor *cupric* copper. This observation, together with conventional thermodynamic considerations relating redox potentials to complexing action (M8) suggest that retardation of the oxidizing action of acutely lethal doses of ionizing radiation could result from chemical stabilization by the protective agents of critical Cu(I) receptor sites, either *directly* through the formation of a dissociable complex or chelate or *indirectly* by electron transfer reactions. Corresponding reactions involving Cu(II) would enhance oxidation of Cu(I).

Irradiation of metal ions in solutions containing complexing or chelating agents has been demonstrated experimentally to favor the formation of that oxidation state which forms the most thermodynamically stable complex with a given ligand. For example, depending on the oxidation state stabilized and the concentration of the complexing agent, ionizing radiation can induce 100 per cent oxidation of ferrous salts or 100 per cent reduction of ferric salts (A9).

This monograph presents biological, radiochemical, and biochemical evidence for a new mechanism of radiobiological action involving aerobic organisms. It is postulated that ionizing radia-

tion produces organic peroxides which damage the genetic structure in the cell nucleus and oxidative processes in the cytoplasm. The cytoplasmic effect is assumed to involve an interference with the ability of copper enzymes—the 4-electron transfer oxidases—from interacting with molecular oxygen because of the radiation-induced oxidation of the cuprous component. Eventually there is produced adverse interactions on the cytoplasmic sites as a result of the nuclear damage and vice versa (D22). Normally, both the cytoplasm and nucleus are irradiated simultaneously in the organism so that a radiomimetic agent which acts only on the nucleus (nucleotoxic agent) or only on the cytoplasm (cytotoxic agent) cannot be expected to fully mimic radiation effects. It would be interesting to test suitable combinations of radiomimetic agents.

A plethora of theories of radiobiological protection and sensitization mechanisms exist (B6, 7, 12, 19, D20, E6, F2a, H12, L6, M1, 21, O2, P2, 4, 9, 17, S31, 44a, T7) which focus principally on radiation effects on the cell nucleus. However, their correlative and predictive value have been singularly disappointing.

The mechanism of radiobiological action described here is called the Cu(I,II)–Peroxy theory. It allows the modifying effects of apparently unrelated chemical agents to be evaluated from a chemical basis and makes it possible to correlate and clarify much of the existing experimental data. New experimental observations and correlations stemming from a preliminary formulation of the Cu(I,II)–Peroxy theory (S21) have already appeared on the action of ionizing radiation on a copper protein (S23), the radioprotective action of cyanide in mammals (S22), and the correlation of comparative radiosensitivity of animal species with copper content (S25).

In view of the central role occupied by copper, not only in the Cu(I,II)–Peroxy theory, but in biology in general (F11), a comprehensive review on selected and new aspects of copper chemistry and biochemistry and in medicine is included in this monograph along with chapters on topics necessary to a fuller understanding and application of the theory.

More and more the important and often unique role of copper in living organisms is becoming recognized. Yet it may be asked,

why should copper be considered to be a key metal in radiobiological action? Research is needed to uncover all the reasons but a few can be enumerated here, though details are given in subsequent chapters.

Copper ions form stronger complexes and chelates than other biologically essential transition cations with the exception of trivalent iron. However, at physiological pH's Fe(III) ions react very strongly with hydroxide ion so that the actual concentration of trivalent iron available for complexing reactions with a specific ligand is reduced by a factor of about 100 million (S20). The net result is that the "effective" constant of copper complexes are often stronger than those of iron and possess remarkable specificity, especially toward sulfhydryl, phenolic, and cyanide compounds.

It has been pointed out that the relative stabilities of Cu(I) and Cu(II) complexes depend much more than do the relative stabilities of oxidation states of other elements differing by one unit on the nature of the anions or other ligands present, on the dielectric constant of the solution, and on the nature of neighboring atoms in a crystal (C11a, p. 749).

Copper ions are uniquely sensitive to steric effects. In the case of the cupric ion, for example, which does not possess spherical symmetry, a remarkable flexibility in reactions is present (Williams, J2, p. 6). In one plane the cupric ion has a radius close to that of nickel and in the plane perpendicular to it there is a close resemblance to calcium.

Because of the enormous varieties of complexing substances present in biological systems no particular correlation between catalytic efficiency and complexing ability exists in such systems. However, as Frieden (F11) has noted, copper possesses unique catalytic properties: "No metal ion or other nonprotein catalyst surpasses copper salts in their versatility as catalysts for an impressive variety of reactions. . . . The recognition of the virtually unique catalytic features of copper ions and copper complexes occupies hundreds of pages in the chemical and the biochemical literature."

Another unique feature of copper is the observation that all the known four-electron transfer oxidases are copper enzymes.

These distinctive enzymes catalyze the reduction of molecular oxygen to water. Not only is copper the principal metal in these terminal oxidases, such as cytochrome oxidase, but *no alternative pathway exists by which the cell can catalytically reduce molecular oxygen to water* (M12). Hence, the copper oxidases provide a unique biochemical function.

The pragmatic applications of the Cu(I,II)–Peroxy theory are, in an empirical sense, independent of speculations concerning the primary biochemical role of copper in radiobiology and cell metabolism or of the specific radiochemical sequences by which organic peroxides are produced in the cell or how they interact to produce the postulated end results. More important, the theory enables the investigator to design experiments which can pinpoint the mechanism or combination of mechanisms by which a chemical protective or sensitizing agent acts.

The modification of radiation injury by radioisotopes deposited in tissues is essentially the same problem as that involved in externally delivered radiation. Indirectly, it is possible to minimize radiation damage from internal emitters by removing them from the body or from critical sites within the body, and thus reduce the radiation dosage (S16).

Chapter II

SELECTED ASPECTS OF COPPER CHEMISTRY

THE LITERATURE on copper, both on its chemical and biological properties is growing rapidly. Much information on the biochemistry of copper now available in relatively recent textbooks and in the literature has become obsolete or is incorrect as a consequence of the discoveries and investigations of the past few years. The extent to which copper is uniquely involved in the functioning of living matter is only now becoming appreciated and uncovered. Some of the general reference material used in the preparation of this chapter are cited here (A3, B8, C4, C11a, C14, F11, G15, M8, M12, M18, O4, S10, S15, S17, S29, S33, S34, and W9) while specific literature citations are given throughout. Emphasis is given to those aspects of chemical behavior of copper ions which bear directly on biological and medical problems.

STABILITY OF COPPER CHELATES

Qualitative and quantitative interactions involving copper, chemical protective and sensitizing agents, and enzymes can be surmised from a consideration of the known interactions involving Cu(I) and Cu(II) and simple molecules. Only the predominating structure of a complex or chelate is discussed. However, when a complexing agent contains several potential ligand atoms, several species of complex ions may and do exist simultaneously. Thus, in glutathione which has α -amine, carboxyl, and sulfhydryl groups, copper may form a chelate ring with the -N, -O pair, or with the -N and -S pair (J2, p. 54). The relative amounts of each form depend on the relative affinities of the cations for the ligand atoms. These, and other factors, such as ligand basicity, hydra-

tion, and pH in affecting chelate stability under physiological conditions have been discussed elsewhere (S15, S20).

Cuprous compounds are covalent and can only exist in water solution in complexed form. In the case of copper compounds in which the cuprous state is more stable than the cupric state, the cupric salt will decompose to the corresponding cuprous salt. Thus, cupric cyanide decomposes to form cuprous cyanide and cyanogen because of oxidation of the anion by Cu(II) to form the more stable though insoluble complex cuprous cyanide. Cuprous sulfate, on the other hand, is immediately decomposed by water to yield cupric sulfate and copper. Part of the reason why Cu^+ is more stable than monovalent ions of other transition elements lies in the fact that the second ionization potential of copper is much greater than the other elements of the first transition series which begins with calcium and ends with zinc (O4, p. 12). The third ionization potential for copper, $\text{Cu}^{+2} \rightarrow \text{Cu}^{+3}$, is very high, which explains why it is difficult to oxidize Cu^{+2} . A summary of the ionization potentials of some ions of biological importance is given in Table II.1. There are other factors which

TABLE II.1
IONIZATION POTENTIALS IN ELECTRON VOLTS (1 e.v. = 23.05 Kcals.)
OF SOME TRANSITION ELEMENTS ^a

<i>Metal</i> <i>Ionization</i> <i>Potential</i>	<i>First</i>	<i>Second</i>	<i>Difference</i> <i>Second-</i> <i>First</i>	<i>Third</i>	<i>Difference</i> <i>Third-</i> <i>Second</i>
Ca	6.11	11.87	5.76	51.21	39.34
V	6.74	14.65	7.91	29.21	14.66
Cr	6.76	16.49	9.73	30.95	14.46
Mn	7.43	15.64	8.21	33.69	18.05
Fe	7.90	16.18	8.28	30.64	14.46
Co	7.86	17.05	9.19	33.49	16.44
Ni	7.63	18.15	10.52	35.16	17.01
Cu	7.72	20.29	12.57	36.83	16.54
Zn	9.39	17.96	8.57	39.70	21.74

^a The Table is adapted from the one given by Orgel (O4, p. 13).

influence ease of oxidation. Thus, one oxidation state may happen to be rendered stable by complex or chelate formation because the energy of complex formation is large enough to compensate for the extra energy needed to produce ionization.

Copper (I) usually forms tetrahedral compounds and copper (II) planar compounds. The coordination number of Cu(I) is

variable; generally it is equal to four but may be only two in linear compounds, while Cu(II) usually has a characteristic coordination number of four but two extra positions may be very weakly coordinated to complete a distorted octahedron (O4, J1).

For most purposes it suffices to consider only the first formation constant because the donor groups in proteins are somewhat fixed in their relative positions and are not usually free to form a consecutive series of complexes, MA, MA₂, MA₃, etc., in the same way as small, freely moving ligands (G15). The tabulated values of stability constants in Table II.2. are useful for deducing which ligand atoms are responsible for binding Cu(II) and Cu(I) with different molecules. Such information is useful for evaluating the "anchoring" propensities of compounds capable of stabilizing the different oxidation states of copper.

Sharp differences exist in the relative stabilities of the complexes and chelates of Cu(I) and Cu(II) as can be discerned from the data compiled in Table II.2. which includes stability constants for Ca⁺⁺ and Ag⁺. Silver is included because its complexes are similar in type and stability to those of cuprous ion and thus serve as a means of estimating the stability constants of Cu(I) when the latter are not known. Copper and silver ions

TABLE II.2

STABILITY OR SOLUBILITY PRODUCT CONSTANTS ^a FOR COMPLEXES AND CHELATES

Compound	Stability Constants (Log Units)					
	Ca	Cu(II)	Cu (I)	Ag(I)	Cu(II)/Ca	Cu(I)/Ca
Alanine (α-)	<1	8	—	—	~8	—
Alanine (β-)	<1	7.1	—	<5	~7	<5
Allyl alcohol	(~0)	—	4.7	1.2	~4.7	(<4)
Acetic acid	0.5	<2	—	0.7	<1.5	(<0.5)
Aspartic acid	0.5	8.6	—	—	8.1	(<5)
Arginine	(<1)	~8	—	—	~8	(<5)
Asparagine	0	7.8	—	—	7.8	(<5)
Azide	—	~2.4	—	~8	—	—
Catechol	(~0)	19.5	~0	—	19	(~0)
Chloride	—	0.1	6.5	9.6	<0.1	~6.5
Cysteine	<1	(~10)	19.2	15	(<10)	~18
Cyanide	(~0)	Unstable	16(β ₂) ^b 24(β ₂)	13.8 18.7(β ₂)	—	16(β ₂)
Diaminodiethyl- sulfide (2:2)	(~0)	9.1	—	7.0	9	—
Diaminopropane (1:3-)	(~0)	9.8	—	5.9	9.8	—
Diethylaminoacetic acid	(~0)	6.9	—	—	6.9	—

TABLE II.2 (Continued)

STABILITY OR SOLUBILITY PRODUCT CONSTANTS ^a FOR COMPLEXES AND CHELATE

Compound	Stability Constants (Log Units)					
	Ca	Cu(II)	Cu (I)	Ag(I)	Cu(II)/Ca	Cu(I)/Ca
Diguanide	(~0)	11.9	—	—	11.9	—
Dimethylglycine (N, N-)	(~0)	9.8	—	—	9.8	—
Dipyridyl (2:2-)	(~0)	17.9(β_3)	14.2(β_2)	6.8(β_2)	—	—
Ethylthioacetic acid	<1	—	—	4.1	—	(~4)
Ethanolamine	<1	~3	—	3.1	<3	—
Ethylenediamine	<1	11	—	4.7	11	—
EDTA	10.7	18.8	—	7.3	8.1	—
Ethylamine	<1	—	—	3.3	—	—
Formic acid	<0.8	~2	—	—	~1	—
Fumaric acid	0.5	2.5	4.0	—	<2	3.5
Glycine	~0	8.1	—	4	8.1	(<4)
Glycine amide	(~0)	5.5	—	—	5.5	—
Glycylglycine	<1.2	6.0	—	<3	(~6)	—
Gluconic acid	1.2	18.3	—	—	17	—
Glutamic acid	1.4	7.8	—	—	6.4	—
Glutathione (oxidized)	<1.4	14.6	—	—	~14	—
Glutathione	<1.4	—	(~18)	—	—	(~17)
Glycollic acid	1.1	2.4	—	—	1	—
Histidine	(~0)	10.6	—	7.4	~10	—
		18.3(β_2)	—	—	—	—
Histidine methy ester	(~0)	9.1	—	—	~9	—
Histamine	(~0)	9.6	—	—	~9	—
Hydroxyquinoline-5-						
Sulfuric acid (8-)	<3.5	12.5	—	—	~10	—
Hydroxyl ion	—	6.8	15	8	~6	~15
Imidazole	0.08	4.2	(6)	—	~4	~6
	—	7.7(β_2)	10.9(β_2)	—	—	—
Iminodiacetic acid	2.6	10.6	—	—	8	—
Iodide	—	Unstable	12	16.1	—	16
Mercaptoethylamine ^a (2-)	(<1)	(10)	(18)	—	(~10)	(~18)
Maleic acid	1.1	<3.9	3.1	—	<3	—
Methylthioethyl- amine	(~0)	5.6	(4)	4.2	~5	—
Malonic acid	1.4	<5.6	—	—	~4	—
Oxalic acid	<3	~6	—	0	<6	~0
Phenol	—	—	—	0.34	—	(~0)
Salicylic acid	0.1	10.6	—	—	10.5	—
Sulfide	—	36	49 ($\beta_{0.5}$)	50 ($\beta_{0.5}$)	—	—
Thiocyanate	~0	Unstable	13.4	12	—	~13
Thiourea	—	—	15.4(β_4)	13(β_3)	—	—

^a The equilibrium constants are expressed as the log of the formation constant for the 1:1 complex: $M + L = ML$; K_1 . In some cases the overall constant, β_n , is given where it represents the reaction: $M + nL = ML_n$; $K_1K_2...K_n = \beta_n$. The equilibrium constants are usually for 25°C and 0.1–0.2 ionic strength. The constants are taken mainly from reference (B23) with additional references from L12, L13 while most of the inorganic constants are obtained from references L7, and M16. An apparent formation constant for copper with mercaptoethylamine has been reported (K17) to be 16.3.

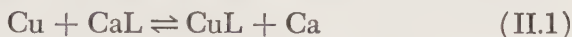
^b The values for cuprous cyanide, $Cu(CN)_2$ are taken from references L7 and V2 respectively. The higher value, $\beta_2 = 24$ at $\mu = 0$, may be more reliable (also reference B23, Part II).

have many points of similarity: both have (I) and (II) oxidation states; their lower oxidation states form linear and tetrahedral structures; and Cu(II) and Ag(II) form planar structures. Generally cuprous complexes are somewhat more stable than the corresponding argentous complexes.

It is also possible to make reasonable estimates of the stability from data on other closely related metal ions. The empirical order of stability of the complexes of bivalent metal ions of the first transition series nearly always follows the so-called Irving-Williams series (II). That is, if the formation constants are plotted against the atomic number of the element there occurs a monotonic increase to a maximum at copper with a drop at zinc, regardless of the nature of the ligand:



The reason for the inclusion of data on the stability constants of calcium is due to the fact that in biological systems a relatively high concentration of calcium ions is present. Hence, the efficiency of copper binding, for example, is a function of the ratio R , of $\log K_1$ of copper to that of calcium which follows from the mass action formulation of the exchange reaction:



If, however, the chelating ligand, L , is also firmly bound by H^+ , for example, the competition by Ca^{++} may be relatively unimportant—a point discussed in the following paragraphs.

In order to estimate the relative stabilities of chelates *in vivo* one must, as a minimum, include the interferences or competition by H^+ and Ca^{++} for the chelating anion and by OH^- for the cation. A simple quantitative procedure for making these calculations is by the application of the *effective constant* (S20). The conventional stability constants may be misleading.

The effective constant is defined as:

$$K_{\text{eff}} = \left(\frac{\alpha_{\text{ML}}}{\alpha_{\text{M}}\alpha_{\text{L}}} \right) K_{\text{ML}} \quad (\text{II.2})$$

where the α 's represent the so-called side reaction coefficients

giving a direct measure of the extent of interfering side reactions. Each alpha term may include the overall effect of several interfering ions. Thus α_L includes the competition of H^+ and Ca^{++} for L but in fact, depending on pH, for example, only one term or the other will predominate. Thus, an important rule exists for simplifying the calculations when several interfering ions are present, namely: The overall alpha coefficients can be taken to be approximately equal to the sum of the individual coefficients. Usually one of the α terms predominate so that the other terms can be dropped.

Consider α_L for EDTA at two different pH's. At neutral pH the concentration of Ca^{++} in the blood is about $10^{-3}M$ leading to a value of $\alpha_{L(Ca)} = 10^{7.6}$ and $\alpha_{L(H)} = 10^{3.4}$. Hence the competition from the H^+ for L can be disregarded. However, at pH = 4 the value of $\alpha_{L(H)} = 10^{8.5}$ and therefore the competition from Ca^{++} would be negligible relative to H^+ at the lower pH. The net result of these effects can be illustrated for the chelates of Cu(II) and Fe(III) with EDTA.

The conventional constant, $\log K_1$ for Fe(III)EDTA is 25.1 compared to $\log K_1 = 18.8$ for the Cu(II)EDTA. However at pH = 7 the $\log K_{eff}(Cu) = 11.2$ compared to only $\log K_{eff}(Fe) = 9.6$. Consequently, despite the fact that the conventional stability constant of the iron chelate is more than a million times more stable than the copper, the actual stability of the copper chelate in the blood can be expected to be much more stable than the corresponding iron chelate of EDTA—a conclusion in agreement with the greater effectiveness of EDTA in promoting the excretion of extraneous copper from the body and in counteracting biological effects of copper. Similarly, in the case of mercury poisoning one would expect that EDTA would be highly effective in view of a $\log K_1(HgEDTA)$ of about 22. However, the value of $\log K_{eff}$ is so small that it would be predicted that EDTA would be ineffective as a treatment for mercury poisoning—in full agreement with the experimental facts.

A full discussion on the calculation of effective constants and their application to biological and medical problems is available (S20).

The relative stabilities of Cu(I) and Cu(II) with different

TABLE II.3

RELATIVE STABILITIES OF FIVE AND SIX-MEMBERED CHELATE RINGS OF COPPER WITH DIFFERENT PAIRS OF LIGAND ATOMS^a

$Cu(I):$	$(-S, -N) \approx$	$(-S, -S)$	$\gg$	$(-N, -N)$	$>$	$(-N, -O)$	$\gg$	$(-O, -O)$
$\sim \log K_1:$	19	15-20		5-8		3-5		1
$Cu(II):$	$(-N, -N) \approx$	$(O, -O)$	$>$	$(-N, -O)$		$(-S, -N)$		$(-S, -S)$
$\sim \log K_1:$	10-20	10-20		7-10		(Unstable)		(Unstable)

^a The log K_1 's are estimated from the data in Table II.2. The designation "unstable" means that the compound in solution spontaneously converts to the cuprous form, through the intrinsic stability may be quite high, and, in special cases, as with copper incorporated in a protein, these "unstable" forms can become stabilized (see K14).

pairs of ligand atoms are summarized in Table II.3. Useful generalizations can be drawn from Tables II.2 and II.3 which have a bearing on the biological specificities of copper ions. These include: (1) Cu(II) forms very stable chelates with ortho hydroxy groupings both in aliphatic and phenolic compounds while no stable Cu(I) compounds of these types exist; (2) Cu(II) but not Cu(I) forms stable chelates with hydroxy carboxylic acids such as citric, tartaric, and salicylic acids; (3) When binding with sulfur is involved, the cuprous copper compounds are far more stable than Cu(II) or any of the other divalent metabolically essential transition elements; (4) The affinity of Cu(I) for nitrogen ligands is less than for Cu(II) except for the nitrogen of the imidazole grouping of histidine; (5) Compounds which contain only one of the two ligands necessary for chelation are generally far weaker (W7). Thus, ethyl alcohol, phenol, or acetic acid form weak complexes with copper as compared to the corresponding *cis*-polyhydroxyl or *ortho*-polyphenol compounds. Similar considerations apply to monothiol as compared to dithiols; and (6) The stability of copper chelates depends on ring size (B8, M8). Five-membered rings are usually more stable than six-membered rings as for example in α -alanine which is more stable than β -alanine. Six-membered rings are often favored, however, when double bonds are present. Four-membered rings containing sulfur and nitrogen as the donor ligands may possess considerable stability as in the case of the N,N-dialkyldithiocarbamates. The stability of seven and eight-membered rings involving copper as in 5-amino-n-caproic acid is far less than the corresponding five and six-membered rings.

Reactions Involving Sulfur Ligands

Copper(I) salts are generally formed when sulfur is the donor ligand and with ligand molecules containing acceptor orbitals which can form dative π -bonds with the d-electrons of cuprous ions as has been pointed out by Orgel (O3), Copper(I) salts only are generally formed with organic thiols such as mercaptans, xanthic acids, thio- and dithio-acids, N-mono- and N,N-di-substituted thiocarbamic acids and N-monoalkyl- or N-monoaryldithiocarbamic acids. *However*, N,N-di-substituted dithiocarbamic acids are able to stabilize cupric copper—a remarkable reaction which has been discussed by Åkerström (A1).

Among the acceptor ligands which stabilize the lower oxidation states of copper by making available acceptor orbitals for π electrons are: $R-N\equiv C$, $C\equiv N^-$, $RC\equiv CR$, $R_2C=CR_2$, $C\equiv O$, $RC\equiv N$, and $R-N=N-R$. It should be noted that in contrast to the complex cyanide salts, the carbon monoxide complexes of Cu(I) are readily oxidized by oxygen (S33).

The order of the stability of sulfhydryl derivatives of poly-amino-polycarboxylic chelating agents and of serum albumin generally parallels the order of solubility products of their sulfides. Thus, it is found that the $-SH$ affinity follows the order: $Ag(I) > Pb(II) > Cd(II) > Zn(II) > Ca(II)$. Gurd and Wilcox (G15) have estimated that the first formation constant of cations with RS^- groupings are as follows (the estimated formation constant is placed in parentheses after the respective cation): $Hg^{II}(>20) > Cu^I(19) > Ag^I(\approx 15) > Pb^{II}(11) > Ni^{II}(\sim 10) > Hg^I(\sim 9) > Cd^{II}(\sim 8) > Zn^{II}(\sim 7) > Co^{II}, Mn^{II}(\sim 5) > Ba^{II}, Ca^{II}, Mg^{II}(\text{small})$.

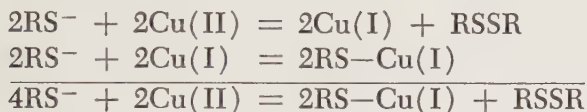
It appears that Cu(I) can complex with the sulfur atoms in a thioether but the strength of the binding is many orders of magnitude weaker than that formed with free $-SH$ groups. Actually Ag^+ and Cu^+ can break thioether linkages—an important reaction which has led to the elucidation of the structure of the cytochrome c molecule (T12).

Cuprous ions reduce disulfide groups under physiological conditions (S48). The reaction of Cu(I) with cystine, for example, proceeds readily according to the following reaction:

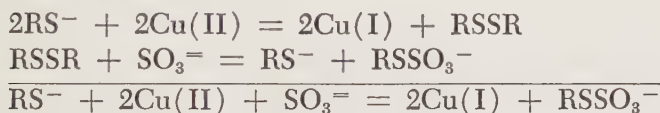


With cysteine, copper has been found (K21, S45) to react as follows:

(a) *Copper-cysteine*



(b) *Copper-cysteine-sulfite*



With excess cysteine the over-all reaction is:



Stable Cu(II)-S bonds in proteins may exist whereas the same bond in a small molecule leads to oxidation of the sulfhydryl group and reduction of Cu(II) to the cuprous state. In small molecules, for example, it is a simple matter for two separate molecules to approach each other and be cross-linked by oxidation of two -SH groups.

Klotz *et al.* (K14), have pointed out in this connection that:

"... With a suitably folded protein, however, the thiol groups need be only slightly below the surface and cross-linking to other molecules could be prevented. Similarly, if the sulfurs of neighboring cysteines of a particular active site are maintained about 3 Å apart, an S-S link will not be possible . . ."

Reactions with Peptides

The binding of copper by peptides merits comment. Of especial interest is the location of the copper binding sites in glutathione and oxidized glutathione respectively. In the case of both Cu(II) and Cu(I) the preferred binding sites in cysteine are the S and N atoms rather than the S and O or N and O. Li showed (L11, W5) that in glutathione the -SH and -NH₂ groups are also the preferred binding sites, as has been found for Zn and Cu(II). However, Cu(II) oxidizes glutathione. In

oxidized glutathione the S atoms form a disulfide linkage and thus it would be expected that the free amino and carboxyl groups would be the primary bonding sites. This assumption is more firmly established by the fact that oxidized glutathione forms a 1:1 complex with the Cu(II) with a formation constant, $\log K_1 = 14.6$. This is close to the value of $\log K_1L_2 = 15.1$ for the 1:2 copper complex of glycine in which it is established that two amino and two α -carboxylate groups are coordinated to one cupric ion.

In actual fact, depending on the metal, the degree to which the sulfur atom in a sulfur-containing amino acid is preferred, varies considerably. An interesting study of this point has been reported by Martin and Edsall (M10). They were concerned with the binding of divalent metal ions to glutathione. They ascertained experimentally, using glutathione and S-methylglutathione, the extent to which the metal ion was attached either to the amino and carboxyl groups of the glutamyl residue or to the sulfhydryl group of the cysteinyl residue. In the case of nickel, manganese and cobalt, only 10, 15, and 30 per cent respectively of the metal was attached to the sulfur atom. In the case of zinc—which would be close to that of copper—80 per cent of the metal was bound to the cysteine sulfur.

While many other elements such as iron, manganese, and zinc, for example, also form complexes and chelates with amino acids, mercaptans, and polyphenols, the rigid conditions existing physiologically impose a high degree of selectivity to copper as has been pointed out by Williams (W7). Thus, the high affinity of iron for the hydroxide ion as well as the extremely high stability of its natural occurring complexes precludes it from reacting appreciably with these compounds at *in vivo* conditions of pH.

In this connection it is interesting to compare the solubility products of the hydroxides of the stable oxidation states of copper with iron and cobalt. These values (C4, p.446) are: Cu^{+2} ($\sim 10^{-19}$), Co^{+3} (3×10^{-26}), and Fe^{+3} (4×10^{-38}). The smaller the solubility product, the smaller is the concentration of available metal ion. It is apparent that Cu^{+2} is more readily available for complexing action than either Co^{+3} or Fe^{+3} .

ABSORPTION SPECTRA

Copper (II) complexes with carboxyl groups show peaks at a wavelength no lower than 700m μ . Copper(II) complexes with electron-donating nitrogens are blue with absorption peaks around 600 m μ . When copper(II) complexes with α -amino carboxylic acids the spectrum becomes sensitive to increasing pH reflecting a shift from solely binding with carboxyl groups to those involving nitrogen (K10). Complexes of copper with α -amino carboxylic acids may shift from about 700 m μ to 600 m μ as the composition of the complex changes from a 1:1 type to a 1:2 type. In general, as the coordination number of copper increases the absorption peak moves to shorter wavelengths. The 600 m μ peak indicates that additional binding of Cu(II) to nitrogen has occurred so that in glycine, for example, at least two bonds are of the amine type.

The absorption peaks of copper proteins parallel those described for the small molecule complexes so that binding to oxygen and nitrogen ligands, respectively, can be distinguished. An additional absorption peak at about 350-375 m μ can be distinguished in some proteins and has been shown to be due to a cupric ion bound to a sulfhydryl (K12).

Cuprous complexes are never blue but are either colorless or pale yellow. They have no strong absorption bands in the ultraviolet as do the cupric complexes.

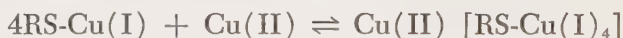
CHARGE-TRANSFER COMPLEXES

"When the absorption of radiation causes an electronic transition between two orbitals such that one is more heavily concentrated on one atom while the other is more heavily concentrated on a different atom, we speak of this as a charge transfer transition and the plot of absorption versus wavelength of light as a charge transfer band or spectrum. The actual degree of charge transfer can, of course, vary from the almost negligible to the almost complete . . . for metal complexes there are often charge transfer bands occurring in the ultraviolet region . . ." (C11a, p. 605).

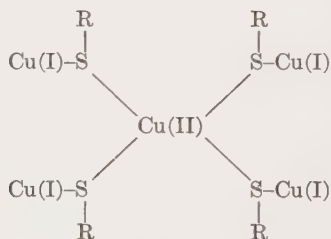
When Cu(II) salts are mixed with thiols the blue color dis-

appears and a colorless or pale yellow color is produced. Depending on the relative concentrations of the thiol and Cu(II), however, the reduction of Cu(II) may first result in the appearance of a violet color which changes to pale yellow. The reverse may result, i.e., when the solution containing the yellow color is allowed to stand in air a violet color may gradually develop.

A careful optical and polarographic study of solutions of copper and thiols was made by Klotz *et al.* (K16). They showed that the violet color resulted from a complex containing copper in two valence states simultaneously. The intense violet color giving an absorption peak at $520\text{ m}\mu$ appears when the mole ratio, Cu(II)/thiol exceeds 0.5—the point at which a 1:2 complex is formed. When the total copper(II) exceeds 0.5 the following reaction is deduced:



A mixed valence complex was represented by the following structure:



It is possible to remove the Cu(II) from the mixed complex with a chelating agent favoring Cu(II) under anaerobic conditions so that the unchelated Cu(I) is not oxidized. In the above complex 20 per cent of the copper is in the +2 state. Within experimental error this was the amount of the blue copper(II) removed from the violet complex (K16).

The formation of complexes containing mixed oxidation states has been demonstrated earlier by many investigators for ions such as tin, antimony, iron, and molybdenum. McConnell and Davidson (M16) have proposed the formation of a strongly colored mixed complex of copper with chloride, namely: Cl-Cu-Cl-Cu .

They suggested that the absorption spectrum be regarded as due to an electron transfer process and that in the ground state the two Cu atoms may be indistinguishable.

OXYGEN-CARRYING CHELATES

An oxygen-carrier is defined as a compound which takes up molecular oxygen and releases it reversibly. The oxygen-carrying proteins of copper and iron are charge-transfer complexes. It is of interest to review a few of the characteristics of synthetic chelates which also act as oxygen carriers (V4). Martell and Calvin (M8) have pointed out that a metal can act as an oxygen carrier when (1) it can exist in two oxidation states and (2) the oxidation of the reduced form of the metal ion does not proceed too far. It is clear that the oxygen-carrying ability of a molecule is related to its ability to catalyze the reduction of oxygen which in turn is a function of the electron donor properties of the metal and the stability of the chelate.

Of historical and practical interest are the bis-salicylaldehydeiminecobalt(II) oxygen-carriers. Various derivatives of these chelates are oxygen-carriers in the solid state as well as in solution in certain solvents (V4). They have been used as a means of producing large quantities of oxygen for welding and other industrial purposes. Some derivatives undergo hundreds of oxygenation-deoxygenation cycles with little deterioration. Irreversible oxidation of Co(II) to Co(III) is the single most important factor in the deterioration of the chelate. The solid oxygen-carriers undergo eventual fragmentation as well because of changes in the crystal dimensions during the cyclizations causing strains to be set up.

In the case of synthetic oxygen-carrying chelates the stability of the metal-chelate bond is critical, otherwise the reaction becomes irreversible either by proceeding too far or else not far enough, because the oxidation potential is too low to promote the necessary weak combination with molecular oxygen. For example, the oxidation potential is usually increased by chelation. In the case of the cobalt(II), the formation of an extremely strong chelate with ethylenediamine increases the oxidation potential to the point where the chelate decomposes water and

combines irreversibly with oxygen (M8, p. 352). On the other hand, the histidine chelate of cobalt acts reversibly as an oxygen carrier in aqueous solution.

The oxygen-carrying reactions may become irreversible for many reasons—not all of which are understood. Some of the mechanisms by which irreversible oxidation may occur include: (1) Gradual loss of the oxidized form of the cation from the chelate because it is much more weakly bound than the reduced form; (2) Hydroxylation of the cation as in the case of ferrous salts in which ferric hydroxide becomes precipitated; (3) Alteration or destruction of the chelating ligand molecules; (4) Changes in the structure of the compound (especially of a protein) so that the diffusion of molecular oxygen into or away from the complex is hindered; and (5) The previously mentioned factor of overstabilization by the formation of too strong a chelate.

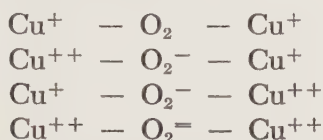
It is apparent that in the iron and copper oxygen-carrying proteins, the protein serves to coordinate with the metal ion in a manner which imparts the delicate balancing necessary for the reversible oxygen-carrying reactions to function. The close similarity between cuprous, ferrous, and cobaltous complexes in relation to their interactions with oxygen, carbon monoxide, nitric oxide and related compounds is discussed in relation to ligand-field theory by Williams (W9) and Orgel (O4).

For many years the only stable non-protein synthetic oxygen-carrying chelates known contained cobalt as the coordinating atom. In 1958 was reported the first synthetic oxygen-carrying chelate, namely iron(II) dimethylglyoxime(V4). It dissolves in aqueous dioxane containing added bases such as pyridine, ammonia, histidine or imidazole which are displaced reversibly by oxygen. The reaction is reversed readily by bubbling nitrogen through the solution. Preliminary reports indicate the existence of a nickel(II) dimethylglyoxime oxygen carrier which functions in strongly alkaline solutions. A quadricovalent iridium(I) complex compound has been reported to react reversibly with molecular oxygen (VI). Though not a synthetic chelate, it has been found that the oxochlororhenate ion $(\text{Re}_2\text{OCl}_{10})^{-4}$ is a reversible oxygen carrier.

The charge transfer oxygen compounds of iron, cobalt, and

copper appear to involve resonance among several possible structures. The first definite proof of resonance equalization of the valencies of the metal ion in a mixed valence compound was shown (E3) for the complex ion $[(\text{NH}_3)_5\text{CoO}_2\text{-Co}(\text{NH}_3)_5]^{+5}$. Paramagnetic resonance studies revealed an equal density of the unpaired electron on each of the cobalt nuclei.

Orgel (O3) has written four simple valence structures for charge-transfer complexes of copper:



He concluded that resonance between the different structures is possible so that no single valency in the complex can be assigned to the copper atoms with certainty.

STABILIZATION OF VALENCE STATES AND CATALYSIS

Metal ions which can exist in two valence states can be stabilized against oxidation or reduction depending on the relative stability of the complex ion formed with the lower and upper oxidation states respectively. The change in oxidation potential is a quantitative measure of the degree to which the particular valence is stabilized. Many excellent papers and books are available which deal with the stabilization of oxidation states (C4, D17, K9, M8, O4).

Cuprous complexes are readily oxidized to the cupric state by oxygen. However, when complexed with ligands such as iodide, cyanide, thiourea, cysteine, etc., the rate of oxidation of cuprous ion is retarded. Similar considerations hold for the reduction of the cupric ion. For example, cupric ions in acid solution are not reduced by agents such as hydroxylamine or hypsulphite when the cupric ion is complexed. However, in basic solution reduction may take place readily if the complex is unstable such as in the case of the ammine complexes of Cu(II).

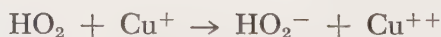
Examples of the role of stabilization in redox systems are described in the following pages because of their importance in understanding the postulated action of protective and sensitizing

agents in modifying radiation toxicity. The effect of complex ion formation on the redox reactions of iron undergoing irradiation has been described elsewhere (p. 82). Some of the important illustrations cited include the role of complex formation in retarding or accelerating the copper catalyzed oxidation of ascorbic acid, and the decomposition of hydrogen peroxides by metal chelates. These examples are of especial interest because they show that a catalytic reaction can be nearly completely inhibited or enhanced depending on the concentration of the complexing agent, the stability of the complex ion, and the particular oxidation state of the metal ion stabilized.

Copper Catalyzed Oxidation of Ascorbic Acid

In neutral or acid solutions the autoxidation of ascorbic acid is catalyzed by copper ions (C4, M8). The cupric ion forms a chelate with ascorbic ion which by an electron shift to the metal becomes a semiquinone. The cupric ion becomes reduced to the cuprous form in the process and dissociates because of the very weak binding of cuprous ion for oxygen atoms. One mole of hydrogen peroxide is formed in this non-enzymatic reaction. During the reaction the copper catalyst appears to undergo a cyclic shuttle from the cupric form to the cuprous form and back to the cupric form upon the oxidation of the cuprous ion and the semiquinone by molecular oxygen. The catalytic action proceeds via the formation of free radicals (C4, M8).

The final step in the series of reactions (C4, p. 43) by which ascorbic acid is catalytically oxidized by cupric copper is as follows:



The regenerated cupric ion is then available to form a chelate with ascorbic acid. Note that the last step of the catalytic reaction shown above requires that the Cu(II) produced by the oxidation of Cu(I) sustain the cyclic series of reactions. Therefore, it is reasonable to expect that stabilization of Cu(I) by complex formation would retard the oxidation rate of ascorbic acid by reducing the rate by which Cu(II) is made available. In the same sense, a corresponding stabilization of Cu(II) should enhance or

stimulate the rate of oxidation of Cu(I) and thus promote the oxidation of ascorbic acid. In summary, then, the presence of complexing agents which form only *moderately stable* complexes or chelates with copper—by “moderately stable” is meant complexes much weaker than that formed by Cu(II) with ascorbic acid itself—the oxidation of ascorbic acid may be accelerated or inhibited.

With the viewpoint expressed in the preceding paragraph as a guide, experiments on the effect of complexing agents on the copper catalyzed oxidation of ascorbic acid were examined. The experiments of Scaife (S6) proved illuminating. He found for reasons which seemed inexplicable, that sodium chloride inhibited the rate of oxidation and glycine stimulated the rate of oxidation. These findings are precisely what would be expected in view of the fact that the chloride ion preferentially stabilizes Cu(I) with a $\log K_1 (\text{Cu}^I) = 6.5$ while glycine preferentially stabilizes Cu(II) with a $\log K_1 (\text{Cu}^{II}) = 8.1$. In general, it was found that the degree of acceleration of the catalysis increases as the stability of the Cu(II) complexes with different amino acids increased.

It must be appreciated that the stimulating or inhibitory action of a complexing agent on the cupric catalyzed reaction is like any reversible chemical reaction. Hence the actual action is a function of the absolute value of $\log K_1$, the concentration of the complexing agent, and the rate of formation of the complex ion. Consequently it is expected that the same complexing agent could conceivably stimulate or inhibit depending on its concentration and value of $\log K_1$. If $\log K_1$ is very high, that is, greater than that of Cu^{II}-ascorbate ($\log K_1 \approx 15$) then a relatively small concentration of complexing agent could inhibit the catalytic action of copper by reducing the copper ion concentration excessively. This would be true whether Cu(I) or Cu(II) was complexed. It is interesting to note, therefore, that the presence of chelating agents which form very strong copper chelates inhibit the copper catalyzed oxidation of ascorbic acid (B30, C4). These chelating agents include EDTA ($\log K_1 (\text{Cu}^{II}) = 18.8$), glutathione and diethyldithiocarbamate ($\log K_1 (\text{Cu}^I) = 18$). If the considerations reported here are correct, very small concen-

trations of a Cu(II) chelating agent such as EDTA should also stimulate, rather than inhibit, the copper catalyzed oxidation of ascorbic acid. The concentrations of EDTA hitherto employed (B30, C4) have been too high to demonstrate a stimulatory action.

In preliminary experiments (unpublished observations of E. Baumgartner) an *acceleration* by EDTA of the copper catalyzed decomposition of ascorbic acid has been found:

The destruction of ascorbic acid in the presence of copper was studied in presence of varying molar ratios of EDTA to a fixed concentration of copper sulfate. At a molar ratio, EDTA/Cu of 2:1, the oxidation of ascorbic acid was nearly completely inhibited. The inhibitory effect of EDTA continued but became less and less as the EDTA/Cu was decreased to 1:1, 1:10, and 1:20. At a molar ratio of 1:40 the oxidation of ascorbic acid was about the same as in the absence of EDTA. However, at a molar ratio of EDTA/Cu = 0.01, that is, 1:100, the oxidation rate of ascorbic acid increased dramatically to values much greater than in the absence of EDTA.

There is another factor involved in the evaluation of the effect of chelating agents on a metal-catalyzed reaction which must be considered in connection with the ascorbic acid system. Neglect of this factor can lead to misleading conclusions as to the mechanism of action of the chelating agent. This factor has to do with the solubilization of the insoluble salt of a metal ion by a chelating agent which may increase the concentration of free metal ions. Several examples can be cited to illustrate this point in connection with the ascorbic acid system. Scaife (S6) has pointed out that the presence of a buffer constituent such as phosphate can reduce the effective concentration of copper ion by the formation of a sparingly soluble salt. In such a system, the inherent retarding action of a cuprous preferring complexing agent such as Cl^- could be masked by an apparent stimulating action resulting from the solubilization of insoluble copper salts. This, indeed, appears to resolve many of the differences observed in the work of Scaife (S6) and Mapson (M5) on Cl^- effects on the copper catalyzed oxidation of ascorbic acid, since the former avoids the use of phosphate. Mapson (M5) has reported both

a stimulating and retarding action of Cl^- depending on the concentration of Cl^- employed.

In the case of a very strong copper complexing agent such as cyanide employed in the presence of phosphate, one might expect a stimulating action at very low concentrations followed by an inhibiting action at higher concentrations.

A careful study of the oxidation rate of ascorbic acid at varying cyanide to copper ratios was reported by Butt and Hollaway (B30). Their results are shown in Figure II.1. They show conclusively that cyanide induces stimulation at lower concentrations and inhibition at higher levels. It was also shown (B30) that a varying azide to copper ratio also produced stimulating and inhibition depending on dose but that the curve was more bell-shaped and less sharp than the cyanide curve—a result to be expected in view of the weaker complexing action of azide relative to cyanide for Cu(I) . It would be worthwhile to ascertain whether the apparent stimulating action of CN^- would be depressed or eliminated if the catalysis by copper were carried out under conditions where no insoluble copper salts are formed.

Decomposition of Hydrogen Peroxide

Copper salts possess a catalase-like activity and therefore serve as a model system for the study of the activity of catalase and oxidases. An excellent review of this subject has been provided by Nikolaev (N3). He points out that the most important factor enhancing the catalase-like activity of copper compounds is the presence of four nitrogen atoms in the sphere of coordination; if oxygen is substituted the activity is decreased. Thus, taking the relative activity of methylamine as 160, that of α -methylhydroxylamine is only 80 while that of alanine is only 0.001.

He also found that modification of the hydrocarbon chains of an amine can increase or decrease the catalase activity depending on the degree to which steric hindrance is involved. The activity of the copper chelate of methylamine is nearly equal to the chelate formed with amylamine, but the chelate formed with dimethylamine, in which the copper is surrounded by a large number of methyl groups, is nearly inactive.

It is postulated (N3) that the catalytic activity of complex

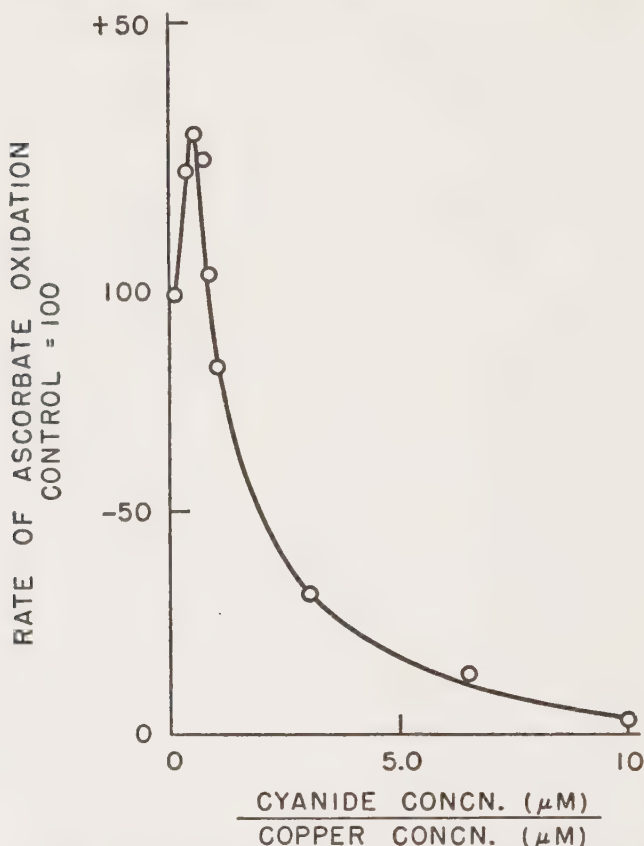


Figure II.1. The relation between oxidation rate and the cyanide to copper ratio (from B 30). The oxidation rate was determined manometrically at 25° C. with 4 mM ascorbate and 1.5 μM CuSO_4 in phosphate buffer (pH 5.5; 0.067 M), in air. Cyanide concentrations were obtained with different mixtures of $\text{Ca}(\text{CN})_2 - \text{Ca}(\text{OH})_2$ in the center well.

compounds of copper is brought about by the entry of two molecules of hydrogen peroxide into the sphere of coordination. This postulate is similar to that reported for the mechanism of action of the catalase-like activity of $\text{Fe}(\text{III})$ -dihydroxytriethylenetetramine by Wang (W4), who suggested that the peroxide anion was coordinated to two adjacent sites in the coordination sites on the $\text{Fe}(\text{III})$ atom. In support of this concept it was demonstrated that the $\text{Fe}(\text{II})$ -tetraethylpentamine chelate which has

only one free position on the metal ion was a very poor catalyst for the decomposition of hydrogen peroxide.

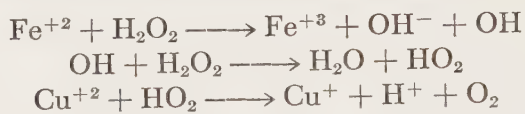
The oxidase-like activity of copper chelates is somewhat dependent on the same factors as those involved in the catalase-like activity. Taking the oxidation of pyrogallol by oxygen as a test system, Nikolaev (N3) reports that chelations of the copper ion with amines increases the oxidase-like activity considerably.

It is of interest to consider here the catalase-like activity of the copper protein, hemocyanin. In a careful investigation of this phenomenon, Ghiretti (G3) found that copper-free hemocyanin did not decompose hydrogen peroxide. However, when the copper-free hemocyanin was reconstituted with *cuprous* chloride the activity toward H_2O_2 was restored. No evolution of oxygen from inorganic copper salts equivalent to those contained in hemocyanin was observed. Ghiretti tested the ability of amino acid complexes to decompose hydrogen peroxide and interestingly found that only arginine gave a complex which possessed catalase-like activity. This is especially interesting in view of the findings of Nikolaev mentioned earlier since arginine is capable of forming a chelate containing one atom of copper to two of arginine in such a manner that the copper is coordinated with four atoms of nitrogen (L10). Further, the hemocyanin molecule is quite rich in arginine which comprises up to 12 per cent of the total amino acids present (G3).

Another interesting catalytic action involving copper is the reaction of human γ -globulin with hydrogen peroxide (P6). Unless cupric ion is present, hydrogen peroxide is without effect. The cleavage reaction of peroxide requires copper specifically and at a ratio about equimolar with that of the protein. The reaction is stopped by EDTA presumably because of the chelation of the copper. Neither nickel or iron are effective in catalyzing the cleavage reaction. The cleavage point may involve those sites in which copper is combined with sulfur since extensive alteration of methionine and cystine residues is observed (P6).

In many instances the catalytic effects of copper are modified by the presence of another metal. These mixed catalyst systems (W9) are well known. An example (U1) of the interaction of copper and iron, for example, in free radical systems is the pro-

motion by copper of the iron-catalyzed oxidation of hydrogen peroxide according to the following reactions:



Chapter III

COPPER IN BIOCHEMISTRY AND MEDICINE

COPPER IS PROBABLY a functional constituent of all cells. There appears to be a close correlation between copper and cellular activity (M7). For example, copper becomes concentrated in the actively growing portions of plants. When radioactive copper is injected into hen's eggs its concentration pattern in the developing embryo resembles that of the metabolic gradient.

From early studies on the copper content of marine organisms (D8) it was concluded that "the amounts progressively diminish as we ascend from the comparatively simple to the more complex forms of life, and that when we come to the fishes, which are provided with hemoglobin, copper persists at a low but rather constant level." From a survey of the copper content of many different forms of life, Brenner (B25) reached a similar conclusion, namely, that "ganz generell der Cu-gehalt des Organismus vermindert, wenn die niederen Tiere sich von der ganz primitiven Stufe zu den etwas hoher differenzierten Formen entwickeln."

According to Cartwright (C3) the adult body contains a total of 100 to 150 milligrams of copper of which the muscle contains about 64 mg, the bones 23 mg, and the liver 18 mg. Some representative values of copper in human tissues are tabulated in Table III.1.

The copper content within a given tissue may vary considerably depending on the particular types of cells involved and the chemical forms of copper. Thus the white and grey matter of brain differ in copper content by a factor of about two. Within the liver several different forms of copper exist. Part of the liver copper is of a relatively inactive "storage type" while other forms of copper in the liver include those which are part of oxidative

TABLE III.1
COPPER CONTENTS OF NORMAL ADULT HUMAN TISSUES*

<i>Tissue</i>	<i>Copper Content (mgm/kgm/Dry Weight)</i>
Liver	20-50
Brain	20-40
Heart	10-25
Kidneys	15-40
Stomach	10-20
Spleen	5-15
Thyroid	~9
Adrenal	5-10
Pancreas	4-30
Bone	~5
Leukocytes†	0.94 $\mu\text{g}/10^9$ cells
Blood serum	1 $\mu\text{g}/\text{liter}$

*Summarized from data given in references A8, B25, C3, K19, S10.

†Within the limits of analytical sensitivity no copper was detected in the lymphocytes, neutrophytes, monocytes or thrombocytes. Most of the copper appeared to be present in the eosinophiles and basophiles (A8).

enzymes, especially cytochrome c oxidase, which is present nearly exclusively in the mitochondria. The mitochondria of liver contain about 8 per cent of the total copper present in the whole organ in rat and man (p. 185). A tabulation of the subcellular distribution of copper and other trace metals in the liver is shown in Table III.2.

In general, the invertebrates contain much more copper than the vertebrates or plants. A rough average value of the copper content of three general groups of phyla illustrating the marked differences in the absolute content of copper is included in Table XIII.1.

COPPER PROTEINS AND ENZYMES

A list and description of the known mammalian copper proteins is given in Table III.3. Blood contains two principal fractions of copper. About 6 per cent is loosely bound to serum albumin while 94 per cent is tightly bound to the serum copper protein, ceruloplasmin fractions. When copper first enters the plasma from the gastrointestinal tract it is believed to be loosely bound to albumin and gradually transferred in the liver to ceruloplasmin. The albumin bound copper is capable of diffusing into the red blood cells, cerebrospinal fluid, urine and across the placenta. When radioactive copper, Cu^{64} , is administered orally or

TABLE III.2
DISTRIBUTION OF TRACE METALS IN SUBCELLULAR FRACTIONS OF RAT AND HUMAN LIVER^a

Rats (5)															
	N	Cu	Fe	Zn	Mo	Mn	Cr	Ni	Cu	Fe	Zn	Mo	Mn	Cr	Ni
			% of reconstituted total							Micrograms of element/mg of nitrogen					
Nuclei	37	30	37	31	28	37	49	—	0.25	2.6	0.77	0.011	0.029	0.0041	—
Mitochondria	18	17	11	9.3	38	39	14	—	.19	1.7	.42	.025	.067	.0029	—
Microsomes	17	4.8	21	11	6.1	14	14	—	.061	3.6	.65	.0050	.032	.0026	—
Supernatant	28	48	32	49	27	9.7	23	—	.39	5.0	2.0	.019	.0072	.0040	—
Reconstituted Whole Liver	—	—	—	—	—	—	—	—	.25	3.3	1.1	.015	.037	.0036	—
Human (1) ^b															
Nuclei	3.1	34	30	25	26	24	50	81	0.57	46	2.5	0.011	0.038	0.065	0.12
Mitochondria	11	7.4	9.7	5.3	14	25	10	4.7	.34	41	1.5	.017	.11	.036	.020
Microsomes	16	4.4	18	8.8	11	8.7	12	0	—	—	—	—	—	—	—
Supernatant	42	54	42	61	49	43	28	14	.68	48	4.5	.017	.051	.027	.016
Reconstituted Whole Liver	—	—	—	—	—	—	—	—	.52	47	3.1	.014	.050	.040	.047

^a Data taken from reference E4. Full details are provided by these authors including standard errors. Results in rats can be compared with earlier studies in T5.

^b Actually two different samples were analyzed. While good agreement between both samples were obtained in general, the results calculated here are from assays on a large sample size (24gm) compared with only 4 gms. in the one omitted. In addition, the smaller sample size was from a patient with a significant anemia, hence the iron content was especially low.

TABLE III.3
MAMMALIAN COPPER PROTEINS^a

<i>Protein</i>	<i>Source</i>	<i>Molecular Weight</i>	<i>Copper Content %</i>	<i>Atoms Copper Per Mole</i>
Cytochrome c oxidase	Beef heart	~93,000	0.07	≈ 1
Cerebrocuprein I	Human brain	30,000–40,000	.029	2
Ceruloplasmin	Human plasma	151,000	0.34	8
Erythrocuprein	Human red blood cells	28,000±2,000	0.32–0.36	2
Hemocuprein	Ox and human blood cells and serum	35,000	0.34	2
Hepatocuprein	Ox liver	—	0.34	—
Tyrosinase	Mouse melanoma	—	0.22	—
			0.25	—

^a Data taken from Ref. S10.

intravenously to humans it appears first in the albumin fraction and four to six hours later in ceruloplasmin (S10, S32).

The physiological functions of the copper proteins remains a problem for future research. However, ceruloplasmin is responsible for most if not all of the oxidase activity of blood and its inherited deficiency is often associated with Wilson's disease discussed later. Tyrosinase catalyzes the oxidation of L-tyrosine to L-dihydroxyphenylalanine (dopa) which then leads to the formation of the melanin pigments. Erythrocuprein appears to be involved in maintaining the integrity of the adult erythrocyte (S32).

When oxygen-carrying proteins have remained extra-cellular, as with the hemocyanins, aggregation into giant molecules has occurred. Frieden (F11) suggests that aggregation diminishes the contribution to the osmotic pressure.

The Four-Electron Transfer Oxidases

A feature of copper enzymes is their ability to use molecular oxygen directly. They constitute a class of electron transfer oxidases which catalyze the reduction of molecular oxygen to water by a four-electron transfer as shown by the following equation (M12):



In the mitochondrial electron transport system copper appears

to be localized at the terminal position as the functional component of cytochrome oxidase (p. 45).

At present four four-electron transfer oxidases are known—all of which are copper enzymes (Table III.4). Of the four, three,

TABLE III.4
FOUR-ELECTRON TRANSFER OXIDASES (G10, M12)

<i>Enzyme</i>	<i>Metal Component</i>	<i>Products</i>
Laccase	Cu	Quinones + H ₂ O
Catecholase function of the phenolase complex	Cu	o-Quinone + H ₂ O
Ascorbic oxidase	Cu	Dehydroascorbic acid + H ₂ O
Cytochrome oxidase	Cu + Fe-porphyrin	Cytochrome c + H ₂ O

the ascorbic acid oxidases, the laccases, and the phenol oxidases, have only copper as the functional group; while the fourth, cytochrome c oxidase, also contains one atom of iron for each atom of copper in the water soluble monomer. The copper oxidases, in the succinct statement of Singer and Kearney (S34) “. . . represent a primitive type of oxidative system in that all carry on a direct transfer of electrons from metabolites to molecular oxygen without passage through a chain-linked mechanism such as the pyridine nucleotide-flavoprotein-cytochrome system.”

The catalytic reduction of molecular oxygen to water has been formally represented as proceeding in a stepwise manner with the consumption of four electrons and four protons. The interesting point is that the stepwise reduction of oxygen to water involves the following free radicals and peroxides (M12): perhydroxyl (HO₂), peroxide anion (HO₂⁻), perhydroxyl cation (H₂O₂⁺), hydrogen peroxide, and OH radical (Figure III.1). Consequently ionizing radiation produces many of the same free radicals and peroxides already involved in the normal oxidative action of the copper enzymes. The following comments of Mason (M12) are of particular interest because of their presumed bearing on the radiobiological role of the four electron transfer oxidases:

“. . . During the formal stepwise addition of four electrons to molecular oxygen, at least two very reactive radical intermedi-

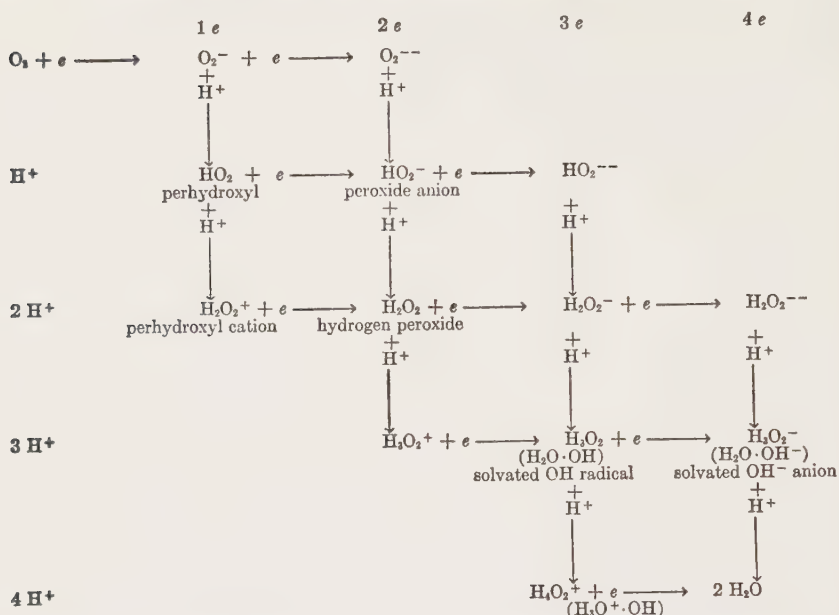


Figure III.1. Formal stepwise reduction of oxygen to water with consumption of four electrons and four protons (from Mason, M12).

ates, perhydroxyl and hydroxyl, are produced. From a biological point of view, reduction mechanisms forming these free radicals are disadvantageous because an energetic random attack upon functioning components of the cell must ensue, and because biochemically efficient utilization of free energy of reaction of these *free* radicals is impossible. The formation and excretion of the third intermediate of oxygen reduction, hydrogen peroxide, is inefficient as a type of respiration compared to reduction of oxygen to water because half of the electron affinity of the oxygen molecule is thereby lost to metabolism. Almost every enzyme which can reduce oxygen to hydrogen peroxide has an alternative and preferred electron acceptor, organized in an electron transfer system ending with four-electron reduction of oxygen. . . ."

Valence Changes in Copper Proteins

The state of copper in copper proteins and the valence changes undergone during their functioning cycles are of fundamental interest. Attempts to demonstrate the changes in valence

have been made by means of chemical tests utilizing the release of copper by glacial acetic acid and the cuprous specific reagent, 2,2'-diquinolyl and electron spin resonance (ESR). Both of these methods must be applied carefully. The chemical test may give erroneous results because of oxidation or reduction of the copper during its release from tissue (p. 87). The ESR studies are only conclusive when a positive signal for Cu(II) is obtained. A negative result, however, can be obtained in samples in which Cu(II) is known to definitely be present. Within these limitations, however, valuable information has been obtained which is cited in the following sections in which individual copper problems are discussed.

In a series of important studies, Malmstrom and co-workers have shown by means of ESR that copper enzymes such as laccase and ceruloplasmin undergo valency changes when functioning catalytically. Other workers have demonstrated valence changes for other copper enzymes. The valency changes undergone by the copper component of cytochrome oxidase during its functioning were demonstrated, using different techniques by Sands and Beinert in 1959 (S5) and confirmed in 1960 and 1961 by others (G13, T1). Further detailed studies by Beinert *et al.* (B18a) indicated that like practically all copper oxidases, "at least 2 copper ions are in close proximity to one another in such a way that they experience a relatively large exchange interaction with one another." They further state that the EPR spectra leave no doubt that the copper in cytochrome oxidase is reduced to cuprous when the enzyme is reduced by substrate. Electron-spin resonance studies of Walaas *et al.* (W1a) indicated a steady-state equilibrium of the cupric-cuprous couple in ceruloplasmin during activity.

Unlike the phenolases, the predominant state of copper is the cuprous form in those enzymes which act essentially as carriers of oxygen (hemocyanin) or which react directly with molecular oxygen as one component in an electron transport system (cytochrome c oxidase). Thus, we have a dichotomy, in which the predominant oxidation state of the copper in the resting enzyme depends on the type of function of the enzyme. In both cases, however, in order for the enzyme to function, a cyclic shuttling

of copper between its two oxidation states must take place. In this manner the systems catalyze a continuous uptake of oxygen.

It is well to distinguish between *oxidation* and *oxygenation*. It appears that the oxygen-carrying proteins such as hemocyanin and the copper oxidases such as cytochrome oxidase form charge-transfer complexes (p. 17) involving either pairs of copper atoms which participate in double resonance or a single copper atom reacting with an oxygen molecule.

In order for oxygenation to occur, electrons from one or both of the Cu(I) coppers must be available to the electron accepting O₂ ligand. However, when the paired copper atoms, for example, are both oxidized to the cupric state then no electrons are available for oxygenation. The deoxygenated forms of copper proteins are especially susceptible to attack by peroxides which cause the copper to be converted to the ionic cupric state—an experimental fact which has been demonstrated with hemocyanins (F4, F5, S23) and cytochrome oxidase (S23, Y1). It has been demonstrated (S23) that the destruction of ionizing radiation of the oxygenation capacity of deoxyhemocyanin is due to the oxidizing action of peroxides.

While we will focus attention on copper it is recognized that other metal ions of variable valence are involved in the sequence of changes induced by ionization in aerobic organisms in various enzymes, though copper is assumed to be most critical. Other essential metal ions existing in two or more oxidation states may be susceptible, namely iron, molybdenum, and cobalt, manganese and vanadium.

The interdependence of metals in biological systems must be recognized. For example, changes in the oxidation state of the copper of cytochrome oxidase influences the oxidation states of the iron atoms present in the sequential cytochrome chain of electron transfer.

Yellow Phenol Oxidases

A group of copper enzymes whose valence relations are of especial interest are the yellow phenol oxidases, which unlike some of the other copper enzymes, do not appear to form stable blue oxygen complexes. These enzymes have two enzymatic ac-

the substrate can become attached to the enzyme. Such ternary complexes involving a metal which serves as a bridge between the enzyme and substrate and the influence of the relative stability constants are assumed to be the intermediate in metal-catalyzed reactions. It has been postulated (S17), that the optimum conditions for the metal ion activation are those in which the stability of the complex formed between the metal and enzyme is approximately equal to the stability of the metal-substrate complex.

Ingraham (11) suggests that in tyrosinase complexes such as cuprylion, Cu O^+ , and percuprylion, Cu O_2^+ are involved. These complexes which presume trivalent copper (C11a, p. 758) are comparable to some iron complexes.

Ceruloplasmin

This is an *alpha*-globulin and is deep blue in color (L8). It accounts for most of the Cu ion and oxidase activity of blood which in turn varies with the serum copper level. The protein has a strong absorption peak at $610 \text{ m}\mu$ and $320 \text{ m}\mu$. These peaks disappear when the copper is removed from the protein or when an amount is present of agent capable of converting half the ceruloplasmin copper to the cuprous form. The copper can be removed by cyanide giving a white, copper-free protein—apoceruloplasmin—which can recombine with cuprous ions to reform the blue ceruloplasmin. The copper is more easily removed when in the cuprous state—thus providing evidence that sulfur is not involved in its binding. Cuprous ion will not exchange with ceruloplasmin copper which is as it should be from considerations of the relative binding affinities for the functional groupings involved which may consist of carboxyl groups and nitrogen. The oxidase activity decreases in proportion to the amount of copper removed from the protein. Its activity is inhibited by Cu(I) complex formers such as KSCN , KCN , sodium azide, and diethyldithiocarbamate.

Of the 8 copper atoms in ceruloplasmin, about 4 are relatively easy to exchange with free ionic copper. Interesting studies on the ceruloplasmin molecule have been carried out by Blumberg *et al.* (B24a) who correlated changes in the optical absorption spectrum with changes in electron paramagnetic resonance and

proton relaxation rates. The fraction of the total ceruloplasmin-copper which is cupric seems to be a function of the history of a particular preparation but it appears that at least 2 to 3 of the copper atoms are cupric perhaps as many as four. All of the chromophoric copper atoms which make ceruloplasmin so blue are cupric. It seems likely (B24a) that this is due to the existence of pairs of copper ions in their ground state in which a cupric ion is connected through a ligand bridge to a cuprous ion in a different ligand field. The ability to undergo charge transfer transition by these cuprous-cupric pairs is said to be very similar to the ferrous-ferric center in Prussian blue.

It is worth noting in connection with the biphasic activities of ions toward enzymes, that low concentrations of anions activate ceruloplasmin while higher concentrations cause inhibition. Similarly, the activity using NN-dimethyl-p-phenylenediamine as substrate, is increased by Fe^{+2} ions and to a lesser extent by Ni^{+2} , Co^{+2} , Zn^{+2} , and Fe^{+3} ions at low concentrations but these same metal ions decrease the activity at higher concentrations (C11)—a frequently observed phenomenon in enzyme studies (D14).

Peroxides can apparently inactivate ceruloplasmin as shown indirectly by the fact that the oxidase activity of serum and purified ceruloplasmin decreases exponentially when irradiated by ultraviolet at 2537 Å (A12). It appears that the UV irradiation causes the copper to become "unbound." As the irradiation of pure enzyme proceeds, the characteristic blue color changes to lighter shades and the change in intensity of color appears proportional to the amount of radiation absorbed. Attempts to reactivate the oxidase activity by incubation with cysteine or ascorbic acid plus copper proved unsuccessful. It was not possible to protect adequately against the effects of UV by prior addition of cysteine, though caution must be used in this experiment inasmuch as the effect of cysteine could vary greatly depending on the concentration used.

Ceruloplasmin from different species of mammals have many properties in common. Especially noteworthy is the fact that regardless of species, the ceruloplasmin contain 8 atoms of copper per molecule. In an interesting study, McCosker (M17) has

shown that the *p*-phenylenediamine oxidase activity of the plasma of different species which appears to be due to ceruloplasmin follows the order: pig, > man, > cattle, dog, sheep, and cat. The ratio of the observed activity is approximately 8:4:2. From these results and others, McCosker suggests:

“ . . . considering the evidence presented here that in human caeruloplasmin 4 of the copper atoms are more labile than the rest . . . it is tempting to suggest that the observed differences in *p*-phenylenediamine oxidase activity in the plasma of various mammals may be a reflexion of the number of catalytically active copper atoms in the molecule of each species.”

Ascorbic Acid Oxidase

The enzymatic oxidation of ascorbic acid by the copper enzyme, ascorbic acid oxidase exhibits a different oxygen stoichiometry than the non-enzymatic reaction catalyzed by cupric ion described earlier (p. 22). In the enzyme reaction no hydrogen peroxide is detected. The net reaction involves the loss of two hydrogen atoms and the uptake of one atom of oxygen per mole of substrate dehydrogenated. This reaction is typical of those catalyzed by the other copper oxidases but is unlike the typical dehydrogenases because only molecular oxygen can serve as a hydrogen acceptor. On an equivalent copper basis the enzyme copper is about a thousand times more effective as a catalyst, and, further the enzyme oxidizes the L-ascorbic acid faster than the D-ascorbic acid which is not observed with the non-enzymatic catalysis. Dawson (D6) has summarized much information on this enzyme.

A solution of the pure enzyme is much bluer than an equivalent amount of aqueous CuSO_4 or even the copper ammonia complex. When a small amount of ascorbic acid is added the blue color disappears rapidly changing to a light yellow. When oxygen is readmitted to the system the blue color slowly returns. This bleaching and recoloring cycle can be repeated several times. The bleaching is characterized by a disappearance of the 606 $\text{m}\mu$ absorption peak.

The activity of the enzyme is inhibited by those agents which stabilize Cu(I) such as cyanide, sulfide, and diethyldithiocarba-

mate. The copper of the enzyme is not exchangeable with ionic copper unless the enzyme is functioning as a catalyst and then only when the enzyme contains cuprous ion. It appears that the primary bonding of the copper is to nitrogen, specifically the imidazole groups of histidine. No free -SH groups are detectable in ascorbic acid oxidase, all the sulfur in the native enzyme is associated with cystine and methionine.

Hemocyanin

The hemocyanins are globulins and occur in the blood but never in the blood cells or tissues of various molluscs and arthropods and are especially striking because of their intense blue color. Because hemocyanins occur in solutions in larger quantities in the readily available circulating fluids, they are a convenient protein for studying the oxygen-carrying properties of copper proteins and their radiation chemistry. An excellent review of the properties and functions of hemocyanins has been written by Redfield (R3) and more recently by Manwell (M4) and Ghiretti (G3, 1962).

In crustaceans 90% of the copper is normally in the form of hemocyanin but in molluscs (*Octopus*) only eight to ten per cent of the copper is accounted for by the hemocyanin. In the latter organisms more than 75% of the total copper present is found in the hepatopancreas, partly as free copper and partly bound organically, while very small amounts are present in all the other organs (G3, 1962). However, even in crustaceans the blood hemocyanin drops almost to zero during molting. At that time 50% of the copper accumulates in the hepatopancreas and the rest is excreted. Seasonal variations in the amounts and size of the hemocyanin molecule have been observed. For example, in *Helix* the size of the hemocyanin molecule is different during the hibernation and estivation periods and has been attributed to the different calcium content of the hemolymph during the two periods (G3).

Each unit of hemocyanin contains one atom of copper with a molecular weight of 25,000 to 37,000. Since two atoms of copper

are required to bind one molecule of oxygen, the minimal molecular weight of the functional unit is 50,000 to 74,000 according to Redfield. These units undergo reversible aggregation and exist as units with molecular weights ranging from 400,000 to ten million and the largest aggregates may contain up to 400 atoms of copper and bind as many as 200 molecules of oxygen.

There does not appear to be any rule as to which organisms have hemocyanin and which have hemoglobin for a respiratory pigment. Hemoglobin, as has been stated by Anson and Mirsky (A10) is not the preeminent heme pigment; it is merely much more widely distributed. Some snails, for example, use hemocyanin, other snails use hemoglobin, and one species, *Buscyon*, employs hemocyanin as a respiratory pigment, yet has hemoglobin in some of its muscles (R3).

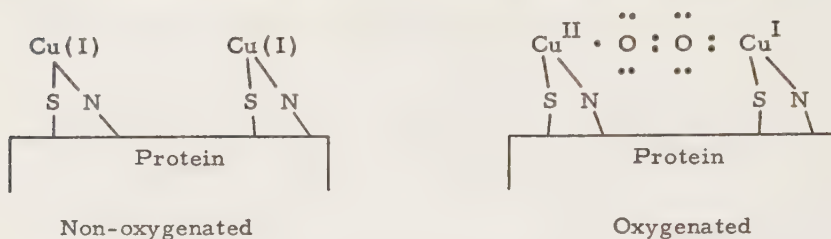
Hemocyanin is not as efficient as hemoglobin in its capacity to bind oxygen. Relative to sea water it does have a significantly higher capacity. For example, sea water can absorb about 0.01 mgm of oxygen per 100 ml while hemocyanin can absorb about 3 to 4 times as much for an equal volume of blood though on the basis of pure hemocyanin the capacity is 25 ml of O₂ per 100 gm of pigment. In molluscs the hemocyanin binds 1 mole of oxygen to a 50,000 equivalent weight of protein while for the arthropods one has 1 mole of oxygen to a 70,000 equivalent. Hemoglobin and hemerythrin are far more efficient in that the protein equivalent to 1 mole of oxygen is about 18,000 (M4).

Hemocyanins from various sources contain from 15.1 to 16.8 per cent nitrogen, 0.8 to 1.2 per cent sulfur, and from 0.15 to 0.26 per cent copper (R3, p. 179). They are nearly the sole protein in defibrinated crustacean blood and large amounts are available directly from the horseshoe crab (*Limulus polyphemus*). These proteins are colorless when deoxygenated and blue-violet when oxygenated. They combine with oxygen in the ratio of two atoms of copper per molecule of oxygen. The oxygen is removed readily by a stream of nitrogen or helium and re-oxygenated by a stream of air. The intensity of absorption per gram of copper is 5 to 10 times greater in oxyhemocyanin than in ordinary complex compounds (K11).

There exist four classes of oxygen carrying proteins: hemoglobins, chlorocruorins, hemerythrins, and hemocyanins. Only the hemocyanins contain copper as the active site while the others are iron-containing carriers (K11). Hemoglobin and chlorocruorin have the metal centered in a porphyrin ring while hemocyanins and hemerythrins do not contain pyrrole prosthetic groups.

From a variety of evidence it appears that the copper in hemocyanin and cytochrome oxidase involves the sulfur of sulfhydryl side chains and the nitrogen of a neighboring histidine imidazole grouping. For example, the formation constant of Cu(I) for cysteine, $\log K_1 = 19.2$ while Felsenfeld (F3) found that the binding of Cu(I) by hemocyanins had a $\log K_1 = \sim 19$. Further, oxyhemocyanin shows an absorption maxima around $600\text{ m}\mu$ which disappear with deoxygenation of the protein or when the protein is treated with reducing agents such as cyanide. Since the $600\text{ m}\mu$ absorption band is characteristic of the binding of Cu(II) to nitrogen ligands of an amine type it is reasonable to assume that the copper in the protein is bound both to nitrogen and sulfur. An absorption band in oxyhemocyanin is also found at about $375\text{ m}\mu$ which according to Klotz and co-workers (K12) represents the binding of Cu(II) to a sulfhydryl group. This is not unexpected because, unlike small molecules, the protein by virtue of the fixed positions of its backbone structure, could provide some stabilization for the normally readily oxidizable Cu(II)-S linkage. The binding of sulfur to copper in hemocyanin has been questioned (M4, p. 194). Two imidazole residues have been suggested. While species differences in hemocyanin may play a role, more and better data on amino acid composition and sequences are needed.

In the case of hemocyanin, the diquinolyl technique indicated that in the deoxygenated protein all the copper was in the cuprous state but that in the oxygenated protein half the copper was in the cupric state. In the case of oxyhemocyanin, Klotz and Klotz (K11) concluded that the copper existed in each of its two valence states simultaneously. The situation can be depicted as follows:



Earlier Williams (W7) had proposed a similar structure on which the combination with oxygen was treated as a charge-transfer complex by analogy to the cobalt oxygen-carrying chelates. In 1941, Rawlinson (R2) in a remarkably astute investigation of the effect of oxidizing agents on hemocyanin wrote:

“ . . . By orientation of certain groups, the protein of haemocyanin probably confers the specific property, on the copper atoms, of combining with oxygen. The haemocyanins of all species so far investigated have been shown to combine with one oxygen molecule for every two copper atoms present. The blue colour of the oxyhaemocyanin might then be caused by the development of a resonating structure when each oxygen molecule is linked between two copper atoms . . . ”

Consideration of the valence changes in hemocyanin upon oxygenation suggest that one of the forms of bound oxygen is the perhydroxyl free-radical ion. Evidence for its actual presence has been described by Klotz (K13). Oxyhemocyanin was added to a solution of benzidine in glacial acetic acid. At 0°C a stable blue color, characteristic of oxidized benzidine was obtained, as would be expected if perhydroxyl radical were released from hemocyanin.

The interpretation given by Klotz and Klotz (K11) has been criticized. One point made by Williams (W8) was that in no-bond complexes in which also charge-transfer forces are involved, as, for example, $\text{Cu(I)} \cdot \text{O}_2 \cdot \text{Cu(I)}$, the structure, $\text{Cu(II)} \cdot \text{O}_2^- \cdot \text{Cu(I)}$ would contribute to the stability so that it would not be possible to detect the Cu(II) in the complex by chemical tests. However, *on the average* half the copper is in the cupric state and when all the copper is released half could appear as cupric unless extraneous reduction or oxidation took place. Manwell (M4) has made a critical evaluation of the views of Klotz and

Williams. He suggests that the existence of resonance systems resolves the problem. Orgel (p. 21) has already postulated such resonating systems.

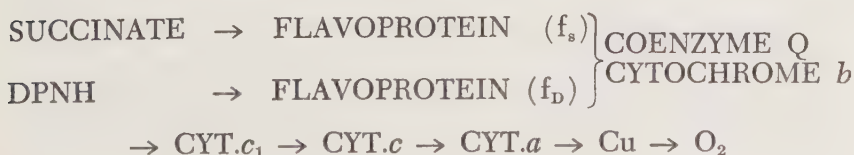
By means of electron spin resonance studies, it is possible to study the oxidation state of copper without the concomitant denaturing effect of the chemical procedure. In the case of deoxygenated hemocyanin or oxyhemocyanin, depending on species from which obtained, no appreciable Cu(II) signal may be found (N1, S36). Unfortunately, at the present time, it is not possible from electron spin resonance techniques to conclude from a negative result whether Cu(II) is present in the protein. A positive signal, on the other hand, does prove that Cu(II) is present. It was found, however, that hemocyanin obtained from *Cancer magister* did yield a signal when the deoxyhemocyanin was titrated with hydrogen peroxide (N1).

Williams (W7) has made the interesting point that the differences between oxygen carrying copper proteins "... may well be no more than a slight change of basicity of the complexing groups. . . ." We have already seen how the strength of chelation affects the properties of synthetic oxygen-carrying chelates.

Cytochrome Oxidase

This enzyme is present entirely in the mitochondria of mammalian cells. It catalyses the oxidation of reduced cytochrome C by molecular oxygen and in the process the oxygen is reduced to water (see pages 6 and 33). Cytochrome oxidase functions as an oxygen-carrying chelate and by a series of alternate oxidation-reduction cycles, a small amount of the enzyme, along with the other cytochromes, participates in the oxidation of relatively enormous amounts of material in living cells.

In the mitochondrial electron transport system copper appears to be localized at the terminal position as the functional component of cytochrome oxidase (G8, G10):



If chronic depression or inhibition of the oxygen-carrying capacity of cytochrome oxidase is induced, the consequences can be serious. For example, in animals moderately to severely deficient in copper or suffering chronic azide poisoning, the cytochrome oxidase activity is depressed to low levels. Death eventually occurs in copper deficient animals even though many enzymatic and metabolic processes including tissue respiration appear unchanged. However, disturbances are found in heme and phospholipid synthesis, osteoblastic activity, succinoxidase activity, and keratin and pigment formation (G1, G2, G14, H18, S26, S27, S10).

The assumption that free radicals are produced by cytochrome oxidase during its catalytic activity is given firm support in the studies of Fridovich and Handler (F10). The aerobic oxidation of sulfite requires free radicals. The nature of the sulfite- O_2 chain reaction has been well-defined (F9). An attempt was made to ascertain the ability of cytochrome oxidase and other oxidative enzyme systems to initiate the aerobic oxidation of sulfite when acting on their respective substrates. Neither cytochrome oxidase nor cytochrome *c* tested separately were able to produce significant sulfite oxidation. However, during the aerobic oxidation of the cytochrome *c* by cytochrome oxidase vigorous sulfite oxidation was demonstrated. Cyanide strongly inhibited the initiation of sulfite oxidation by this system.

According to the investigators (F10), the results suggest that the reduction of oxygen by cytochrome oxidase proceeds by successive univalent steps yielding oxygen radicals. Electron spin resonance studies by several different investigators, especially those of Sands and Beinert (S5) and Commoner (C11) have demonstrated the presence of free radicals in cytochrome oxidase systems during the catalytic process.

Excellent reviews and articles on the biochemistry of cytochrome oxidase are available (G12, G13, K8, L17). This literature serves as a key to the voluminous material available on a still controversial subject. Part of the controversy results from the difficulty of obtaining purified enzyme without altering the sensitive copper-protein bond or accessibility of the copper. King (K8) has well expressed one aspect of the experimental problem:

"... Lipid probably forms an integral part of some of the cytochrome components and the only means of extracting them found so far involves the use of detergents such as deoxycholate or digitonin, which form micelles with the lipid component. The "solutions" obtained by such means may permit of physical separation of some components and certain enzymic properties are retained; but it would be rash to assume that either the physical or enzymic properties of such "soluble" preparations are identical with those of the components of the intact mitochondrion itself . . ."

Probably the most reliable and best preparations of cytochrome oxidase are reported in the papers of Griffiths and Wharton (G11, G12) which provide the best information to date on the preparation and properties of purified cytochrome oxidase. Their enzyme preparation (G12) existed in solution as a polymeric aggregate of varying molecular weight (0.2 to 1×10^6). They report a copper to heme *a* ratio of about 1, and it appears that preparations which contained a copper to heme *a* ratio of nearly 2 were contaminated by ionic copper.

It is of interest to observe the change in the distribution of copper and cytochrome *a* during the preparation procedure. In Table III.5 is shown the relative activity of cytochrome oxidase and copper to cytochrome *a* ratios in the original mitochondria preparation obtained from beef heart and in the most active concentrate of the enzyme prepared by Griffiths and Wharton

TABLE III.5
DISTRIBUTION OF COPPER AND CYTOCHROME *a* IN MITOCHONDRIA
AND IN PURIFIED CYTOCHROME OXIDASE^a

Sample	Cytochrome ^a <i>mμ</i> moles mg protein	Copper <i>mμ</i> moles mg protein	Activity ^b <i>Q</i> ₂	Cu Cytochrome <i>a</i>
Mitochondria	1.18	3.0	8,500	2.54
Mitochondria after dialysis against 0.01M KCN	1.52	1.47	12,000	0.97
Purified cytochrome oxidase	8.2	9.2	55,000	1.12
Purified enzyme after dialysis against 0.01M KCN	7.9	8.2	57,500	1.04

^a Data are taken from reference G13.

^b The *Q*₂ (μ l of O₂ per mg of protein per hour) and corresponds to μ moles of cytochrome *c* oxidized per mg of protein per minute at infinite cytochrome *c* concentration.

(G13). Also shown is the copper-cytochrome ratio in the same fractions which had been dialyzed against to remove extraneous copper—that is, copper presumably not bound or part of cytochrome oxidase. It is seen that the copper-cytochrome *a* ratio is the same in the mitochondria and purified enzyme after dialysis against cyanide. This suggests that a fraction of the copper present in mitochondria is non-functional as far as cytochrome oxidase is concerned. This is an important point to consider when correlations between mitochondrial copper levels and radiobiological effects are attempted (p. 184).

The copper in cytochrome oxidase does not appear to be bound to the iron-containing heme *a* moiety. The experimental basis for this observation is reported below (from G13):

“ . . . Copper in purified cytochrome oxidase preparations is not extracted by acid acetone (0.025 N HCl in 99% acetone)—a reagent which removes heme *a* quantitatively from cytochrome oxidase. Copper is found in the protein residue after this treatment and can be liberated by further treatment of the residue with 50 per cent (volume for volume) acetic acid with no liberation of heme *a* into the supernatant solution . . . ”

The results just cited do not preclude the possibility, as Griffiths and Wharton (G13) point out, that copper and heme *a* are linked in a functional manner in intact cytochrome oxidase. It is conceivable, considering the complex chemistry of copper, that the flow of electrons from copper to cytochrome oxidase to cytochrome *a* proceeds through a bonding of the copper to the sulfur of a cysteine residue and one of the nitrogens of a nearby histidine or arginine residue—a supposition suggested by the work of Tuppy (T12) on the amino acid sequence in cytochrome *c*.

The effect of various inhibitors on purified cytochrome oxidase is shown in Table III.6. It is of interest to note that all of the cuprous preferring chelating agents inhibited the enzyme. The lack of inhibition by the cupric preferring agents such as EDTA and 8-hydroxyquinoline could be due to difficulty in obtaining suitable contact between these compounds and the enzyme-bound copper. It has been demonstrated that the copper in cytochrome oxidase is bound in such a way that it is not readily

TABLE III.6

INHIBITION OF PURIFIED CYTOCHROME OXIDASE BY CHELATING AGENTS^a

Chelating Agent ^b (Sodium Salts)	Oxid. State Cu Preferred	Conc'n	Inhibition
		mM	%
EDTA	II	1.0	0
8-hydroxyquinoline	II	1.0	0
Diethyldithio-carbamate	I	1.0	25
Azide	I	1.0	66
		5.0	90
Cyanide	I	0.005	78
		0.01	100
Thioglycolate	I	0.1	65
		1.0	76
BAL (2, 3 - dimercaptopropanol)	I	1.0	44
		2.0	78
Sulfide	I, II	0.1	30
		1.0	90

^a The data are taken from the report by Griffiths and Wharton (G12). See text for description of the enzyme and its preparation.

^b The inhibitors were added to the standard manometric assay systems containing 0.05 mM cytochrome *c*.

available for chelation and that the copper, in fact, is not released readily by dialyzing with chelating agents. In the case of EDTA the high water solubility may prevent a sufficient concentration from entering the relatively non-polar environment in which the copper is presumably bound, while the insolubility of 8-hydroxyquinoline plus its shape may drastically reduce the concentration available for reacting with copper.

The marked depression of cytochrome oxidase activity in copper deficient animals is discussed later. It is significant that cytochrome oxidase activity is unaffected by iron deficiency in animals and can only be restored by copper administration. The loss in activity of cytochrome oxidase is correlated with copper removal during dialysis against KCN (W4a). *No iron loss occurs as a result of the dialysis treatment.*

The *in vivo* inhibition of cytochrome oxidase has not received sufficient study. It would be of interest to determine the *in vivo* inhibition in different tissues as a function of time following administration of a given agent. The inhibition of cytochrome oxidase by β -aminoethylisothiuronium (AET) in rat liver homogenates has been measured by Zins *et al.*, (Z1) and it was found that 59 per cent inhibition was attained with a concentration of

$2 \times 10^{-3}\text{M}$. The administration of 200 mg/kg of AET to rats thirty minutes before sacrifice had no effect on the cytochrome oxidase activity. However, considering the fact that the *in vivo* concentration of the AET would be only $2 \times 10^{-3}\text{M}$ if evenly distributed assuming no losses from metabolic activity, it appears unlikely that an inhibition in the liver, at least, would be observed. The injection of 100 mg/kg of D-penicillamine to mice apparently had no effect on the cytochrome oxidase activity of the liver and neither did the intraperitoneal injection of 950 mg/kg of cysteine hydrochloride when measured within five minutes after injection. (S24). It is probable that AET, cysteine, and penicillamine do inhibit cytochrome oxidase *in vivo* but that the enzyme is reactivated during the preparation of the tissues for analysis and/or the inhibiting agents are destroyed (see Chapter VIII, p. 139).

It has been found (S22) that the administration of 2 mg/kg of sodium cyanide to mice, corresponding to a concentration of $4 \times 10^{-5}\text{M}$ of cyanide if evenly distributed, produced about 10 per cent inhibition of liver cytochrome oxidase. A dose, when measured within ten minutes after injection, of 5 mg/kg or 10^{-4}M if evenly distributed, produced about 40 per cent inhibition under the same conditions. In view of the very marked inhibition by very small concentrations of cyanide (Table III.6), the observed *in vivo* inhibition, even assuming only a small fraction of the injected cyanide reaches the enzyme sites, is quite reasonable.

The measurement of the *in vivo* activity of cytochrome oxidase following the administration of agents such as cysteine is very important but at the present time may not be possible with present techniques (p. 87). Aside from this point it would be of interest to determine the *in vivo* inhibition in different tissues as a function of time following administration of a given agent. It may be that data on the reduction of oxygen tension in the bone-marrow of mice following the intraperitoneal injection of KCN are related (M21, 1961). Following a dose of about 5 mg/kg of KCN the oxygen tension remained reduced for about 30 minutes compared to about 15 minutes after a dose of 2.5 mg/kg. What relation if any these data have on the protective effect of cyanide is discussed in Chapter VII.

The effect of peroxides on cytochrome oxidase activity is es-

pecially interesting in view of their production by ionizing radiation and their radiomimetic actions (W11). In unpublished studies using purified cytochrome oxidase, it has been found that H_2O_2 causes irreversible changes in the enzyme. The activity could not be regenerated with dithionite. When added to tissue homogenates both benzoyl peroxide and succinic acid peroxide inhibited cytochrome oxidase to the extent of roughly 50 per cent at peroxide concentrations of 10^{-4}M (S24). These must be considered minimum values. Peroxides produced *in situ* would presumably be inhibitory at lower concentrations (see p. 72).

Wills (W11) has carried out an extensive series of studies on the enzyme inhibitory properties of the radiomimetic compound, succinic peroxide, $(\text{O}\cdot\text{CO}\cdot\text{CH}_2\cdot\text{COOH})_2$ which, in aqueous solution immediately hydrolyzes to the per-succinic acid, $(\text{HO}\cdot\text{OCO}\cdot\text{CH}_2\cdot\text{COOH})$, the active form. Among the enzymes tested by Willis was cytochrome oxidase. He claimed that 10^{-3}M succinyl peroxide did not inhibit. However, in experiments in which succinyl peroxide was added to mouse liver homogenates, considerable inhibition of cytochrome oxidase was observed (S24). The results obtained were as follows: $5 \times 10^{-3}\text{M}$ — 82%; 10^{-3}M — 27%; and at 10^{-4} — 7%. The 50 per cent inhibition point proved to be $3 \times 10^{-3}\text{M}$.

Exposure of linoleic acid or methyl linoleate to UV, ionizing radiation, or simply by the bubbling of air through a solution, results in the production of peroxides (B20, C9, M17). Clubb and Wills (C9) have demonstrated that peroxides were produced in linoleic acid in borate buffer after small doses, e.g., 1,000 r, providing the dose rate was reduced to 25 r an hour. Bernheim *et al.* (B20) showed that incubation of washed tissue suspensions or of mitochondria with ascorbic acid inactivates several enzymes including cytochrome oxidase. Methyl linoleate which had been irradiated with UV also inhibits cytochrome oxidase. In all cases, the inhibition of cytochrome oxidase was shown to parallel the amount of oxidized fatty acids or peroxides formed as measured by the thiobarbituric (TBA) reagent (B20).

Ottolenghi (O6) has studied the interaction between ascorbic acid and mitochondrial lipids and has shown that mitochondrial suspensions do not form lipid peroxides upon incubation but that rapid peroxide formation does take place when relatively

small amounts of ascorbic acid are added to the incubation mixture. The rate of lipid peroxide formation in the mitochondrial-ascorbic acid system is increased by ferrous ions much more so than by ferric ions, no test of copper ions was made. Inhibition of peroxide formation in the system occurs upon the addition of EDTA. Peroxide formation in the absence of ascorbic acid was induced by the addition of large amounts of ferrous salts. It is interesting to note that incubation of nuclei relatively free of mitochondria and microsomes yields negligible amounts of peroxide even when relatively large amounts of nuclei are incubated in the presence of ascorbic acid or ferrous salts (O6).

The effects of irradiation on the activity of cytochrome oxidase are discussed in Chapters IV and XIII.

COPPER DEFICIENCY

The essential nature of copper in mammalian physiology has been demonstrated by several observations, especially in animals made copper deficient either experimentally or naturally as a result of their environment. Resulting metabolic disturbances are seen in many ways: iron metabolism, phospholipid synthesis, osteoblastic activity, and keratin and pigment formation (M6, M7, S10).

As the copper deficiency continues, specific symptoms reflecting individual metabolic defects appear in turn: the growth rate of the young animal decreases; the hair becomes harsh and if pigmented loses its color; anemia and other signs of impaired iron metabolism appear, demyelination of the central nervous system frequently originates in the fetus and may appear in copper deficient adult animals but is not often seen because the animals die before the nervous symptoms appear (M7). These symptoms are curable only by administration of copper but not iron.

Marston (M7) has commented upon the sequential nature of copper deficiency in sheep. As the copper reserves become depleted he emphasized the fact that "... an ordered sequence of distinct symptoms appears, as if, one by one, different metabolic

processes become adversely affected, each in its turn, through partial impairment of the particular enzyme system which governs it."

Many enzymes and metabolic processes in copper deficient animals remain unaffected. Of further interest is the fact that a decrease in liver catalase and reduced glutathione in the liver is observed aside from the pronounced reduction in the levels of cytochrome oxidase. These decreases do not occur in iron deficient animals.

A chronic reduction of cytochrome oxidase activity induced by copper deficiency leads to demyelination of the nervous system (G2, H18, M7). Demyelination has also been produced in sheep by administration of potassium cyanide and in monkeys by the administration of either potassium cyanide or sodium azide (H19). Vitamin E deficiency which results in elevation of peroxide levels also leads to demyelination (M7). Very small doses of cyanide induce lesions of the central nervous system without necessarily producing demyelination (S38a).

The first symptom to appear in copper deficient sheep is a disturbance in the process of keratinization. The crimp, a characteristic feature of the wool, disappears but can be returned immediately if the copper concentration of the blood is increased or if the copper is applied directly to the skin. The disturbances in the character of the wool fiber and in its pigment are associated directly with the follicles. Histochemical studies have demonstrated that the lesion is related to free -SH groups in certain intracellular structures and that the copper catalyzes the oxidative closure of the thiol residues of the prekeratin fibrous protein to the disulfide linkages of keratin (M6, M7).

The above mentioned pathological effects of copper have not been demonstrated with certainty in man. Scheinberg (S10) points out that the reason is due to the fact that the daily dietary supply of man (2-5 mgm/day) is high relative to the body levels (75-150 mgm) and that the loss of copper is low. Actually, the ratio of dietary supply to body content for copper is ten times higher than that of iron, hence it is not surprising that iron deficiency occurs despite the fact that the total amount of iron in the body amounts to 4000-5000 mgm. It is reasonable to assume,

however, that copper is essential in man from the animal observations and from the functions of the copper enzymes.

A condition in which the control of copper balance is defective is known as Wilson's disease (S10) and appears to be related to a hereditary deficiency of ceruloplasmin, the copper protein. The pathological effects of the disease are manifested long after the deposition of excess copper in the tissues. As times goes on, the excess copper leads to pathological changes in several organs, especially in the liver, brain, and kidney and eventually causes death. Later in this chapter, further discussion of Wilson's disease is given and it is proposed that the primary biochemical lesion is caused by chronic depression of cytochrome oxidase activity induced by excessive amounts of copper incorporated in the mitochondria.

The single most striking effect of copper deficiency in animals is the large decrease of cytochrome oxidase activity (G1, G2, G11, G14, H18). The activity of the liver cytochrome oxidase is impaired early in copper deficiency and increases with copper depletion. As much as 76 per cent of the cytochrome oxidase activity is lost when the overall copper content of the liver is reduced to about half the control level. The cytochrome oxidase activity of the brain and kidney is also reduced. The activity of the enzyme is regained upon the feeding of copper to the animals. It would be important to determine the actual depletion of mitochondrial copper specifically in relation to the decrease in cytochrome oxidase in the liver.

It has been found that copper reduction has no harmful effects in animals until inhibition of cytochrome oxidase activity occurs. In the brain, for example, degenerative changes do not occur until the copper content drops below a critical threshold of 2.6 $\mu\text{gm/gm}$ dry weight at which point the enzyme activity begins to drop (M25). Ataxic symptoms occur when cytochrome oxidase activity is inhibited.

It is interesting to note that in copper deficient animals, the tissue respiration appears unchanged (p. 46) despite the depressed activity of cytochrome oxidase. The reasons for this apparent lack of correlation of respiratory activity and cytochrome

oxidase activity are obscure. The work of Lundergårdh (L17, p. 351) on the respiratory mechanisms in yeast are at least suggestive in this respect:

“. . . Even inactivation of a large portion of the cytochrome oxidase by 80% CO does not appreciably lower the respiration in starved yeast. The DPN-linked enzymes are here playing the role of a pacemaker. This situation is characteristic of higher plants, too. At high activity of the DPN-linked enzymes, e.g., in yeast fed with glucose, oxygen takes over the role as a pacemaker. The relation between oxygen tension and respiration and between oxygen tension and percentage oxidized cytochrome oxidase is here of first order . . . The common belief that the effectivity of the cytochrome oxidase conveys a comparatively pronounced insensitivity to variations in the O_2 -pressure is thus valid only regarding systems with low or moderate power of dehydrogenation. In many plant tissues, according to the low percentage of cytochrome oxidase, the O_2 -tension is of considerable influence. To what extent the b-shunt to oxygen compensates for this shortage in a_3 is yet incompletely known . . .”

COPPER IN MEDICINE

A great many diseases are associated with alterations in the concentration of the blood copper proteins (S32). The occurrence of low serum copper levels (hypocupremia) is less frequent than increased serum copper levels (hypercupremia). Conditions associated in the human with hypocupremia include newborn infants, sprue, nephrotic syndrome (caused by loss of ceruloplasmin in urine), Wilson's disease, and the nephrotic syndrome. It appears that hypocupremia is due to a decrease in the concentration of ceruloplasmin.

Hypercupremia occurs in numerous conditions. These include acute leukemia, pregnancy, Hodgkin's disease, iron deficiency in infants, infection, aplastic anemia, chronic leukemia, hyperthyroidism, and hemochromatosis. In these conditions the hypercupremia, according to Shields *et al.* (S32), is due to an increase in α_2 globulins other than ceruloplasmin. Since an increase in serum α globulins usually parallels an increase in cerulo-

plasmin it is concluded that hypercupremia is a nonspecific reaction and is associated in humans with tissue damage from any cause.

Wilson's Disease

This disease arises from abnormal copper metabolism. Excessive amounts of copper are found in the tissues, especially the brain and liver. Death often occurs and the results of treatment have been mixed, and even contradictory. The symptoms and clinical signs of Wilson's disease are described below (S9, W3):

These may appear suddenly or develop gradually, and not necessarily in the same sequence in all patients. A common detectable abnormality is a hereditary deficiency of ceruloplasmin. Another early sign is an elevation of the concentration of non-ceruloplasmin plasma copper and of copper in the liver. High concentrations of copper are found in the brain, and kidney and other tissues.

It is rare for the disease to appear before the age of eight, after which the disease may progress so rapidly that death occurs by the age of ten or eleven. The illness develops in young adults and patients may survive into the fourth or fifth decade.

In infants and young children the symptoms may be those of liver disease. In adolescents tremor is commonly the initial symptom. As the disease progresses muscular rigidity and contractures come to dominate and emotional and some mental deterioration may occur. Concurrently with these changes there is an inability to close the mouth so that the face has a fixed grin, and drooling becomes constant. Beside the typical tremor, evidence of chronic liver disease is apt to be noted and the patients may die either from progression of the basal ganglia lesion, from liver failure, or following hemorrhage from oesophageal varices.

Other changes in Wilson's disease include bony changes, and the corneal Kayser-Fleischer rings. These brownish rings appear to be due to copper deposited at the corneal margin.

It must be kept in mind that in Wilson's disease, unlike in copper deficiency, the pathological effects of excessive deposits of a metal are superimposed upon those symptoms postulated to be associated with a common biochemical lesion. The obvious

secondary effects associated with excess copper are found in similar conditions involving other heavy metals. These include liver cirrhosis, renal dysfunction, hemolytic anemia, jaundice and aminoaciduria. Similarly, animals which ingest excessive amounts of copper over long periods exhibit similar symptoms.

The rationale for treatment of Wilson's disease now in vogue is to diminish the absorption of dietary copper by diet and drugs (potassium sulfide) and to promote the excretion of copper by means of chelating agents of which BAL, EDTA and penicillamine are the most prominent. However, the actual fraction of the total tissue copper removed by treatment with chelating agents is insignificant. As a matter of fact, the efficiency of removal of copper generally diminishes as treatment proceeds (S8).

The inability of these regimes to prevent the deposition of excess copper in the tissues is emphasized by the results of treatment of very young children. For example, (S9), one patient age ten months was found to completely lack plasma ceruloplasmin. His sister, who had also lacked ceruloplasmin, developed Wilson's disease at age twelve. Even though the ten month old was treated as if he had Wilson's disease, by age three an analysis of a biopsy specimen of his liver showed a content of more than 250 microgms. of copper per gm. wet weight compared with a normal concentration of about 7 microgms.

The efficacy of treatment has been disappointing. Nevertheless, many dramatic recoveries of patients treated with chelating agents have resulted. Evaluation of the effectiveness of therapy is complicated by the fluctuating course of the disease. Even in those patients showing improvement at first with various treatments, general deterioration gradually occurs (S8). The clinical course noted in patients receiving chelating agents does not seem to parallel the cupriuria. (B17, S29, p. 302).

Discounting those nonspecific symptoms and biochemical changes in patients with Wilson's disease caused by tissue deposits of heavy metals, one is impressed with the fact that patients with Wilson's disease and animals suffering from copper deficiency may share symptoms in common. Brain lesions, however, do not appear to be related.

In animals the specific symptoms of copper deficiency

vary from species to species and do not appear full-blown because the animals die before all the symptoms become manifested as is the case with the neurological disturbances (M7). The neurological disorders often give rise to ataxia or spastic diplegia usually associated with regional degeneration of the myelin of the nervous system. The skeletal changes resemble those found in scurvy. In dogs (B15, B16) the marked bone changes found show no disturbance in the metabolism of calcium or phosphorus. It is significant that these signs and symptoms associated with copper deficiency are curable only by copper administration and not by iron or other metals.

A suggestive parallel in the symptoms common to Wilson's disease and copper deficiency involving the skeleton have been commented upon by Finby and Bearn (F6) who made a careful study of the roentgenographic changes found in the skeletal system of twenty patients with Wilson's disease. Of the twenty patients fourteen showed major skeletal abnormalities. Many of the skeletal changes resembled those described by Baxter and co-workers (B15, B16) in copper deficient dogs. According to Finby and Bearn (F6):

"... copper is essential, either directly or indirectly for normal bone formation since a deficiency of copper in dogs results in thin, malformed bones which show a lack of normal mineralization and trabeculation. It is not immediately apparent how these observations in animals are related to the present findings in patients with Wilson's disease in whom an increased total body copper is ordinarily found . . ."

They point out, further, that it is unlikely that all the skeletal abnormalities are related to the disturbed renal function but rather represent abnormal copper metabolism.

New concepts of the pathogenesis and diagnosis of Wilson's disease are presented here along with suggestions for new approaches to therapy. These new concepts are based on the speculation that the primary biochemical lesion in both Wilson's disease and in copper deficient animals is the inactivation of mitochondrial enzymes. The inactivation, as with other biphasic actions, can occur with too little or too much of a metal. In the

case of Wilson's disease it is suggested that the inactivation results from the incorporation of excessive copper within the mitochondria. In view of the fact that much of the copper in the mitochondria is associated with cytochrome oxidase, it is postulated that it is one of the critical enzymes inactivated. It has already been mentioned in this chapter that a chronic depression of cytochrome oxidase activity is observed in copper deficient animals. It is interesting to note that chronic depression of cytochrome oxidase, whether induced by chronic administration of cyanide or by copper deficiency produces similar lesions in the brain (H18).

The pathogenesis of Wilson's disease is postulated to involve a transfer of copper from the tissues to the mitochondria. Considering the rapid turnover of mitochondria (F7, S49, page 74), it is easy to see that the reconstitution of individual mitochondrion in a milieu containing high concentrations of available copper would eventually lead to the incorporation of copper within the mitochondria. Histochemical studies of copper localization in tissues from patients with Wilson's disease show the presence of copper in both the cytoplasm and nuclei (U2, U3). However, high tissue levels of copper over short periods of time do not necessarily mean high cellular levels. Immersion of tissue in solutions of copper salts, for example, does not result in cytoplasmic deposition of copper (U2).

Presumably part of the excessive mitochondrial copper becomes bound to functional groups on the mitochondrial enzymes which may not be directly involved in the enzyme function. However, as the number of copper atoms attached to the protein increases, a critical point is reached at which the activity of the enzyme would be expected to drop rapidly as is depicted in Figure III.2.

In preliminary experiments (S24), the effect of varying concentrations of copper on the activity of cytochrome oxidase in mouse liver homogenates was determined. The experiment was carried out by adding known amounts of cupric chloride to mouse liver homogenates before assaying for cytochrome oxidase activity by a spectrophotometric method using cytochrome *c* as substrate. When the amount of copper added approached

the amounts found in patients with Wilson's disease, namely about 200 micrograms per gram fresh weight, the activity of the enzyme began to drop rapidly. At a concentration of 1 mg of copper per gram fresh weight, the inhibition amounted to 40 per cent and remained at 40 per cent even when 5 mg/gm of copper was added.

It would appear reasonable from these experimental observations to expect that in a patient with Wilson's disease, the presence for several years of about 200 micrograms of copper or more per gram fresh weight could lead to a chronic inhibition of mitochondrial enzymes.

The above concept of the pathogenesis of Wilson's disease clarifies certain anomalous or obscure aspects of the disease. Thus, the time relations involved in the development of the disease would be governed by the time necessary for the mitochondrial rather than the total tissue copper levels to reach critical values.

It becomes understandable why injection of large amounts of copper salts to animals does not produce the over-all picture of Wilson's disease (S29, p. 294). It is unlikely that copper injections lead to an accumulation of excessive copper in the mitochondria since relatively long times extending over weeks or months would presumably be required to raise and maintain the mitochondrial copper levels. This does not rule out an uptake

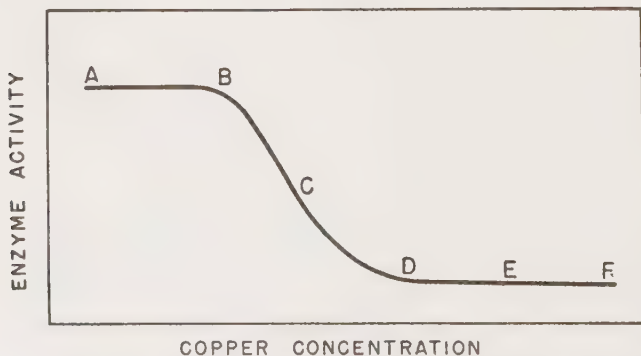


Figure III.2. Assumed variation of the activity of copper oxidases within human tissues with copper concentration. See text p. 61 for discussion of points marked by letters.

of copper for short periods by normal mitochondria, but the turnover time may be high as has been observed following administration of manganese salts (C2, C12).

It is apparent that the usual analysis for total tissue copper in Wilson's disease does not reflect the amount of biochemically active copper present in the mitochondria. Therefore, according to the concepts presented here it is necessary that these mitochondrial levels be determined. The techniques of differential centrifugation in sucrose solutions for fractionation of tissues in order to make the analyses on subcellular fractions of liver and other tissues have been applied by Thiers and Vallee (T5) and Edwards *et al.* (E4). The type of data shown in Table III.2 should be obtained in human controls and patients with Wilson's disease.

It is obvious that, according to the concepts presented here, the efficacy of copper removal therapy discussed below cannot be ascertained from total tissue copper levels but by changes in copper levels in the mitochondria. Other diagnostic tests could measure the cytochrome oxidase activity in the tissues of patients with Wilson's disease though this may not generally be practical because of the large amounts of tissue needed. Other possible tests include the measurement of the oxidative capabilities of the isolated but intact mitochondria in the presence of suitable substrate or cytological examination of the mitochondria which might reveal degenerative changes (D7, L16).

Treatment of Wilson's disease would appear to require the removal of excess copper from the mitochondria. The success of treatment, however, would depend on the absolute amounts of copper accumulated within the mitochondria. It can be expected that the amount of copper removal necessary for improvement would vary from patient to patient. The variation can be explained by reference to Figure III.2. At point F, removal of copper corresponding to an amount at point E would be without effect while removal of the same amount beginning at point D would result in an appreciable restoration of the enzymatic activity. Whether success would attend complete removal of extraneous copper from the mitochondria depends on whether that the disease had not produced irreversible changes in the mito-

chondria or the organism. Hence, patients whose disease is in an advanced stage, that is, those with severe lenticular degeneration and widespread destructive lesions in the brain, might not respond as favorably as those who suffer from what Bearn terms the "pseudosclerotic variety" of the disease (B17). It is also clear from Figure III.2 that the patient may be relatively unharmed despite the accumulation of copper in the mitochondria in the region from A to B, but that beyond this point the disease could progress rapidly.

It is conceivable and even probable that in the early stages of chelating agent therapy that the mitochondrial copper levels in liver and other tissues might increase despite some net loss of copper from the tissue as a whole. In some patients treated by Bickel, Neale, and Hall (B22) the Kayser-Fleischer ring and skin pigmentation *increased* despite prolonged treatment with BAL and an increased excretion of copper. It has been observed experimentally, as well, that the administration of a single dose of chelating agent, cysteine, to an animal administered a trace element, cobalt, caused an increase in mitochondrial levels even though the tissue as a whole accumulated less of the metal as a result of the administration of the chelating agent (M15). It has been pointed out that chelating agents may increase the fraction of the metal which is biochemically active and thus cause the metal to be transported to sites it would not ordinarily enter to any extent (S18). It is conceivable that an increase in biochemically active copper may follow treatment in patients with Wilson's disease—at least in the early stages. This may explain the exacerbation of the neurological symptoms often observed in patients during the early stages of treatment (S8). It appears that in one case at least, the use of BAL hastened the death of the patient (B22).

Pharmacological factors are, of course, of great importance (A3, A10, S18). Thus, if a chelating agent cannot contact the tissue deposits of copper it will be ineffective or of decreased effectiveness. Similarly, compounds which are catabolized or otherwise metabolically altered so that they lose their chelating properties, cannot be effective. In general chelating agents which contain sulfonic acid groups or other highly polar groupings tend

to remain extracellular and will be less effective. On the other hand, compounds possessing higher lipid solubility are usually more toxic insofar as absolute doses are concerned but not necessarily in terms of the therapeutic index.

Based on the concepts of the pathogenesis of Wilson's disease described in this chapter it is worthwhile to explore specific approaches to therapy. It is assumed that in all cases, the usual restrictions of copper intake in the diet are followed.

1. *Lake-forming Dyestuffs and Chelating Agents*

The first step is to immobilize or render less soluble the tissue copper so as to minimize or prevent transfer of copper to mitochondria. In principle this can be accomplished by the use of lake-forming dyestuffs which act by forming insoluble chelates—polychelates—with copper. This approach has proved, experimentally, to be more effective in the case of beryllium poisoning than the use of treatment with the usual soluble chelating agents (S18). Chemically, the dyestuffs potentially the most useful would be the triphenylmethane dyes with ligand atoms so placed as to be capable of forming five or six membered chelate rings with copper. The groupings involved would be hydroxyl-carboxyl combinations as in salicylic acid, or amino-carboxylic acid groupings or sulfhydryl types. In any event these dyes would have to be able to form lakes at neutral pH.

Following the immobilization of the tissue deposits of copper it would appear that intensive treatment be instituted with soluble chelating agents. Two types of chelating agents are the alpha-amino carboxylic acids and sodium salicylate and derivatives. One of the most effective is the metabolically stable penicillamine (p. 168). Actually the compounds with -S, -N, ligand donor atoms offer the best possibilities.

2. *Molybdate-sulfate Feeding*

It has been observed that the symptoms of copper deficiency are produced in animals whose diet is high in molybdenum even though their copper intake is normal. Along with the development of the signs of copper deficiency there occurs a concomitant depletion of tissue copper, especially in the liver. The use of sul-

fate along with molybdenum appears to increase the rate of copper loss (M24). It would be of interest to test the addition of a molybdate-sulfate combination as a prophylactic in children who are aceruloplasmic but have not yet accumulated excessive copper in the tissues, and as a treatment in patients with the usual symptoms of Wilson's disease. It would be necessary in such studies, however, to determine the optimum dose of molybdenum and the amount of sulfate to be added relative to it because the degree to which copper is removed is highly dependent on dose. As a matter of fact, in some species molybdenum feeding may even result in deposition of copper. Information and literature references on the effect of different levels of molybdenum and sulfate in relation to copper metabolism is given in the article by Miller and Engel (M24).

Molybdate feeding as a therapy in Wilson's disease has been attempted (B22). In four patients, molybdate in daily amounts of from 5 to 20 mgm per kgm body weight were fed daily without sulfate while a supplement of 5 gms of sodium sulfate were given to two of the patients for temporary periods. The molybdenum was given for four to as long as eleven months. Two of the four patients showed no improvement while one showed improvement during nine months of molybdenum therapy and relapsed rapidly when molybdenum was withheld. The molybdenum therapy caused a sustained increase of excretion of copper amounting to about 50 per cent in the urinary copper and 100 per cent in the fecal copper.

The above studies are merely suggestive, and in view of the marked dependence of copper removal with dose of molybdenum and sulfate it is unfortunate that the investigators did not study the effect of varying molybdenum-sulfate doses. It is possible that improved therapy could be obtained by means of molybdate feeding, possibly combined with administration of a non-molybdenum reacting chelating agent. As a prophylactic agent Mo appears especially promising.

The mechanism of action of molybdenum on copper is obscure. High dietary levels of other metals, such as manganese, zinc, and nickel, sometimes limit copper storage as well in some

species (M24). The experimental evidence indicates that molybdenum makes copper unavailable for metabolism, e.g., molybdenum decreases markedly the amount of copper transferred to the fetus through the placenta of the ewe (M7). Added copper can overcome the effects of molybdenum. It is tempting to consider the possibility that molybdenum and possibly zinc may be involved, presumably enzymatically, in the excess absorption of copper from the gut in Wilson's disease (B17, W3). The molybdenum enzyme, xanthine oxidase as described by Tiunov (T8), is present in appreciable concentration in the intestinal mucosa (S42) and other tissues of man. Whether this enzyme is involved in some manner in the passage of copper say, through chemical interaction between copper and the enzymatic reaction products would be worth investigating.

3. Mitochondrial Modification and Stimulation of Cytochrome Oxidase Synthesis:

The mitochondria undergo changes under different metabolic conditions (D7, L16). One of the changes include swelling during starvation. It is conceivable that penetration by chelating agents would be facilitated during the period the mitochondria are in a swollen state. Perhaps the administration of chelating agents or molybdate feeding during this stage would hasten the removal of mitochondrial deposits of copper. Another means of altering mitochondria *in vivo* is by a *scorbutic* diet (D7). In this case the mitochondria fuse to form large bodies—the chondriospheres. It is possible assuming maintenance of the patient's well-being that alternate periods of starvation and feeding or a scorbutic diet could accelerate the normal release of copper or cause some reactivation of cytochrome oxidase because of the general stimulation of mitochondrial metabolism resulting from the re-establishment of the normal structures.

It is interesting to speculate that cytochrome oxidase synthesis might be stimulated by administration of certain hormones. For example, the serum concentration copper of the protein, ceruloplasmin, is increased in normal individuals following administration of estrogen (R7) ethinyl estradiol. Whether this increase occurs simultaneously in other copper proteins is unknown.

Treatment of patients with Wilson's disease by administration of estrogen has been attempted (B17, S29, p. 300). The increase in ceruloplasmin was generally negligible in patients with low or moderately low serum ceruloplasmin and copper levels. However, patients with normal ceruloplasmin showed a marked chemical response marked by a rise in ceruloplasmin and serum copper. One of the patients showed remarkable improvement.

The function of most copper enzymes is unknown. It is possible that aside from cytochrome oxidase other copper enzymes such as cerebrocuprein, hepatocuprein, and erythrocuprein may be involved in the production of Wilson's disease.

It is apparent that the elucidation of the biochemical roles of copper will lead to marked advances in our understanding of cell metabolism and human disease. Successful treatment and management of Wilson's disease depends on the acquisition of such information.

Cancer and Aging

It has often been speculated that endogenously produced free radicals and peroxides may be involved in carcinogenesis and aging (H6, M2). Jensen (M2, p. 164), for example, has pointed out that most agents which are mutagenic—x-rays, UV, nitrogen mustards—are carcinogenic as well.

It has been demonstrated that peroxides are mutagenic (p. 94). In view of the known actions of ionizing radiation on DNA, deoxyribonucleic acid (B7), it is interesting that Dickey (M2) suggested that the fundamental biological action of mutagenic agents is a free-radical promoted depolymerization of DNA.

Harman (H6) has suggested that since aging appears to be accelerated by ionizing radiation, that an increase in the concentration in the organism of compounds such as mercaptans capable of reacting rapidly with free radicals might decrease the rate of attack on constituents such as DNA, and thus lead to a decrease in aging. He presents data which suggests that the feeding of anti-oxidants to mice results in an increase in the half-survival time. He further speculates that part of the effect of aging could be due to a decrease in the level of anti-oxidants in the body with increasing age. He cites data (H5) which

showed a decrease in the serum concentration of mercaptan groups in normal men with age, e.g., at age 20-40 the level was 55 μ moles per 100 ml of serum while at age eighty the level was 40 μ moles per 100 ml.

It is tempting to speculate that part of the aging process involves the inactivation of part of the activity of cytochrome oxidase by the endogenously produced peroxides acting on the cuprous component of the reduced form of the enzyme (S21). The gradual loss of energy utilization by the cell mitochondria is presumed to be deleterious to the organism. If this speculation is correct, then it should be possible to slow down the aging process by administration of agents capable of reactivating the fraction of the cytochrome oxidase lost through the normal metabolic processes. Such agents would presumably be those capable of reducing oxidized copper back to the cuprous state, i.e., the cuprous preferring complexing agents. In this connection should be noted the suggestive findings of Harman (H6) that the addition of agents such as cysteine and 2-mercaptoethylamine to the diet of mice prolonged the half-survival time. Apparently no change in food consumption was involved because the weight gains of control and treated mice were the same.

Antipyresis and Hypothermia

It has been pointed out elsewhere (R5, S19) that the antipyretic actions of salicylates and analogs appears to be due, at least in part, to their ability to chelate with copper. It is of further interest to note that antipyretic agents derived from salicylic acid and aminopyrine are cupric preferring agents. Consequently, it was of interest to ascertain the corresponding effects of cuprous preferring agents. The studies of Hope (H13) revealed that the radioprotective thios were all hypothermic. This finding leads to speculations that cuprous preferring agents might possess an antidotal action against salicylate poisoning as well as a general antagonistic action toward some of the effects of salicylates. In fact, it has been demonstrated recently by Erlenmeyer and associates (E7) that the cuprous preferring agent, 2-amino-4-(6-methyl-2-pyridyl)-thiazole, antagonizes the analgesic activity of salicylic acid.

Chapter IV

PEROXIDES AND COPPER OXIDASES IN RADIOBIOLOGY

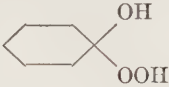
BIOCHEMICAL ASPECTS OF PEROXIDE FORMATION AND DISPOSITION

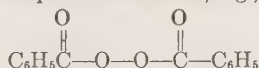
ORGANIC PEROXIDES are considered to play a more active role than hydrogen peroxide in the biological damage produced by irradiation (B7, L6, F2a). The experimental basis for this consideration is discussed in this chapter. While hydrogen peroxide is capable of producing damage, especially in cell suspensions, in contrast to the intact mammalian organism, the efficient decomposition of H_2O_2 by catalase tends to minimize its effects. Organic peroxides, on the other hand, are by and large unaffected by catalase (B9).

Chemically, most peroxides can be classified into the six groups (M9) listed in Table IV.1. Other types of peroxides include polymers formed by the action of oxygen on conjugated olefins or vinyl monomers; transannular peroxides of terpenes, sterols, and polynuclear aromatic hydrocarbons; and the polyalkylidene peroxides such as the dimeric and trimeric acetone peroxides. It is well to note that organic peroxides are, in general, most stable in neutral or slightly acid aqueous solutions.

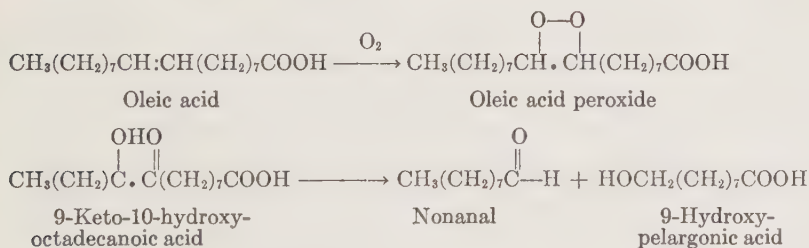
The oxidation of unsaturated acids takes place preferentially at the double bond. Susceptibility to oxidation becomes greater with an increase in the number of double bonds (D9, p. 156). Thus the readiness of attack of some unsaturated acids is as follows: linolenic > linoleic > oleic. The position of the double bonds play a role; acids with conjugated double bonds are more active than are the corresponding non-conjugated compounds. The re-

TABLE IV.1
 ORGANIC PEROXIDE CLASSIFICATIONS^a

Type	Example	Analog
Hydroperoxide Dialkyl ^b	C_2H_5OOH $C_2H_5OOC_2H_5$	C_2H_5OH $C_2H_5OC_2H_5$
Peroxy acid (or per- acid)	$CH_3\overset{\overset{O}{\parallel}}{C}-OOH$	$CH_3\overset{\overset{O}{\parallel}}{C}-OH$
Peroxy ester (or per- ester)	$CH_3-\overset{\overset{O}{\parallel}}{C}-OO-\overset{\overset{CH_3}{\mid}}{C}-CH_3$	$CH_3-\overset{\overset{O}{\parallel}}{C}-O-\overset{\overset{CH_3}{\mid}}{C}-CH_3$
Diacyl ^c	$CH_3-\overset{\overset{O}{\parallel}}{C}-OO-\overset{\overset{O}{\parallel}}{C}-CH_3$	$CH_3-\overset{\overset{O}{\parallel}}{C}-O-\overset{\overset{O}{\parallel}}{C}-CH_3$
Peroxy derivatives of aldehydes and ketones		 (Hydrated form of cyclohexanone)

^a From Martin (M9).^b Diaralkyl peroxides form a part of this class, e.g., the peroxide of hexaphenyl ethane, $(C_6H_5)_3C-OO-C(C_6H_5)_3$.^c Diaroyl peroxides form a part of this class, e.g., dibenzoyl peroxide,

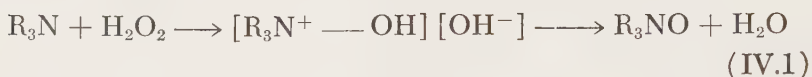
actions of unsaturated acids with oxygen result in peroxide formation in the first stage and later cleavage and other changes occur as shown for oleic acid (D9).



Reactions of Peroxides with Amines

The direct oxidation of tertiary amines to the oxide by peroxides and hydrogen peroxide is a general reaction with few limi-

tations (C16, W2). In the case of H_2O_2 , the reaction is formulated as follows:



The reaction of benzoyl peroxide with dimethyl aniline follows a similar course (W10, p. 591). Secondary amines react readily with benzoyl peroxide yielding chiefly benzoic acid and o-benzoylhydroxylamines. Primary amines give a complex mixture of products.

The effects of substituents on the antioxidant efficiency of aromatic amines in autooxidation reactions can be correlated (W2, p. 435) in a most remarkable way by use of the Hammett sigma function from the Hammett equation (H3):

$$\log k/k_0 = \rho\sigma \quad (\text{IV.2})$$

which states that the ratio of the rate or equilibrium constant for a substituted benzene derivative to that of the unsubstituted derivative of the product of two parameters, ρ , which is specific for the reaction and σ , which is determined by the nature of the substituent. It would be worthwhile to explore the application of this function to radiobiological processes.

Peroxides and Metal Ions

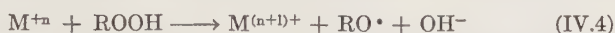
Some of the best known catalytic actions are those in which trace amounts of heavy metals are involved as, for example, in the Haber-Weiss mechanism for the decomposition of hydrogen peroxide by ferrous ions (U1). Scholes and Weiss (S13) demonstrated that the degradation of nucleic acids by x-rays or by chemical action involving H_2O_2 in the presence of Fe^{+2} were remarkably similar. The ferrous ions generate OH radicals which are postulated to degrade the nucleic acid, S by the following reaction (S13):



In the absence of ferrous ions, hydrogen peroxide produced barely detectable degradation, as was demonstrated in the case

of adenine (S13). Similarly, other workers have demonstrated the catalytic decomposition of nucleotides by peroxides in the presence of trace metals such as cupric ion (B26).

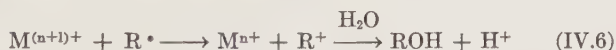
The literature on metal catalyzed autooxidations is quite voluminous (T10, W1). According to Walling (W2, F2a, p. 10) many transition-metal ions capable of undergoing one-electron changes of valence oxidize peroxides producing OH radicals with hydrogen peroxide and RO• radicals with organic peroxides:



The resulting metal ion may be rereduced either by oxidizing peroxide to a peroxy radical:



or by attacking a carbon radical:



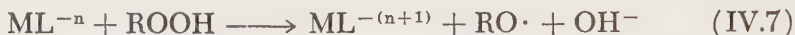
In reactions such as these the metal ions are recycled so that traces only are required. Walling points out (F2a, p. 11) that similar schemes involving metal-containing enzymes have often been suggested.

It would appear obvious that agents which inactivated *both* oxidation states of metal catalysts presumably present in critical volumes of an irradiated organism or in the surrounding solution would reduce radiation damage. Brintzinger, Prijs, and Erlenmeyer (B26) have pointed out that chelating agents could serve as prophylactic agents against radiation by inactivating the metal ion catalysts. In these cases chelating agents, lake-forming dye-stuffs, or precipitating agents (which often involve chelation) could conceivably minimize the organic hydroperoxide and organic peroxides attack on critical molecules. However, chelating agents which appear to be extracellular are ineffective radio-protective agents (Chapter V).

In principle and in actual fact, chelating agents can render metal ions more active biologically (S15) and, for example, result in enhanced production of peroxides or molecular degradation. In principle this can be accomplished by making trace metal ions available to a given volume of tissue or solution. For example, a chelating agent can serve to transport otherwise insoluble

salts making available metal ions which would otherwise be unavailable.

A metal chelate, ML, can itself generate peroxy radicals as has been demonstrated (M20). Consequently, the following reaction becomes possible:



The rate constant for this reaction, as exemplified by one involving iron(II) EDTA and cumene hydroperoxide is independent of pH over a wide range (M20).

It is of interest to recall the work of Kuhn and Wasserman (K22) who observed that the rate of production of oxygen from H_2O_2 by Fe^{+3} was about 100 times more rapid with the α, α' -dipyridyl complex of *ferrous* ion. The enhanced catalytic action of the chelated iron was due to the preferential formation of the very stable ferrous chelate, thus preventing ferrous ion from acting as a chain terminator (U1).

Peroxides and Cellular Components

The very high yield of organic peroxy radicals resulting from irradiation of lipids and lipid-containing material is especially significant when it is considered that they are found in mitochondria and even in the nuclei of many types of functional cells (H4). Of the total dry weight of the mitochondrion, 30 per cent consists of lipids characterized by fatty acids residues with a high degree of unsaturation (G7)—a situation especially conducive to the formation of peroxides by a chain reaction. There exists an intimate contact between mitochondria and lipid droplets. In some cases a lipid droplet is surrounded by a sheath of mitochondria (N4, p. 382). These observations suggest another means by which peroxides in ingested fat can be transferred to mitochondria and vice versa.

It is well to note in the case of layers and emulsions of fats and fatty acids that while the hydrophilic groupings are dissolved in the water phase of the cell, their long hydrocarbon chains are so oriented that they are essentially free of water and can be considered pure hydrocarbons insofar as their radiation chemistry is concerned. A model of the mitochondrial membrane has been

carefully developed by Tedeschi (T3). His results suggest that mitochondria possess a semipermeable membrane composed, at least in part, of a bimolecular lipid layer, in which the internal monomolecular layer is convoluted (Figure IV.1).

In many cases, the peroxides found in the water phase can presumably diffuse into the hydrocarbon phase. Obviously, factors such as the thickness of the membrane through which diffusion must occur must be considered. In this connection, important information on the nature of the mitochondrial membranes and their dimensions has been summarized by Sjostrand (S35).

The long life of radiation produced organic peroxide, plus the rapid turnover of the mitochondria suggest that cytoplasmic peroxides can easily contact mitochondrial structures. For example, despite the very low mitotic index of the liver cells, it has

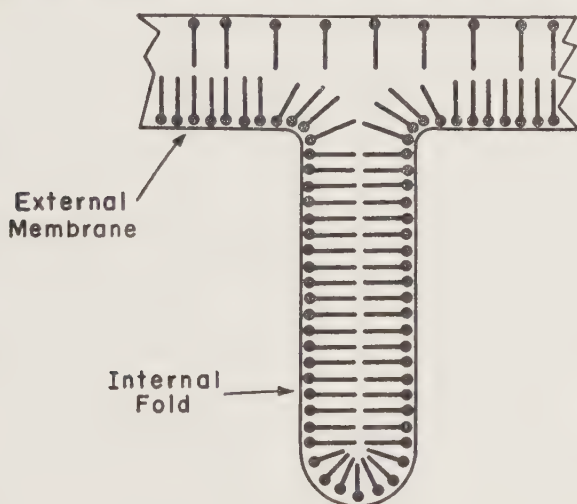


Figure IV.1. Model proposed by Tedeschi (T3) for the molecular structure of the lipid component of the mitochondrial membrane. The model consists of a bimolecular lipid layer where the inner monomolecular layer is convoluted—

- , Polar end of molecule
- , Non-polar part of molecule

The bimolecular arrangement shown by the model is considered to be enclosed by two protein layers, consequently the convolutions would correspond to cristae and be continuous with the surface membrane.

been found that the mitochondria are very labile. When measured by the incorporation of free guanidine —C¹⁴ arginine into protein arginine of rats continuously exposed to isotope, the half-life of rat liver mitochondria was found to be about three days, while that of the total, mixed proteins of rats similarly treated was two and one-half days (S49). Other studies (F7) based on the turnover rates of labeled protein, lipid, and cytochrome of rat liver mitochondria suggest that mitochondria are broken down as a unit with a half-life of about ten days, while an apparently more metabolically active component appeared to have a half-life of roughly two days. It is also worth noting that the experiments of van Bekkum (B18) suggest that mitochondria *in vivo* undergo biochemical changes shortly after irradiation and that the cytochrome system is involved. Further discussion of mitochondrial turnover, number, and oxidative metabolism is given in Chapter XIII.

The ability of the peroxy radicals to react with the copper site in the mitochondrial enzyme, as well as the possibility that the local oxygen concentration is higher at this site is suggested by considerations of the nature of the environment. An interesting discussion of these points in relation to the interaction of peroxide ions with oxygen-carrying proteins has been given by Klotz *et al.* (K14). Part of his discussion bears directly on the probable situation existing for the copper oxidases:

“. . . the nature of the environment of the active site, whether polar or nonpolar, may influence the stability of the ionic structure of the oxygenated form. A nonpolar environment, by tending to reduce the local effective dielectric constant, would stabilize ionic association. Thus the peroxide ion would tend to stay on the protein instead of diffusing into the aqueous phase. Furthermore, a nonpolar environment on the protein would tend to increase the local concentration of dissolved oxygen. It is not often realized that the solubility of O₂ is much greater in organic solvents than in water . . .”

The above considerations suggest that peroxy radicals generated in the cytoplasm may be effective inhibitors of mitochondrial enzymatic function as well as those peroxides produced within the lipid phase of the mitochondria. Further, peroxides produced

within the mitochondria may become available for interaction with the cell nucleus.

The interesting experiments of Daniels (D2, D3) provide suggestive evidence for the role of mitochondria and the cytoplasm in radiation toxicity. He demonstrated that the injection of non-irradiated whole protoplasm as well as the "heavy" portions of centrifuged amoeba into supralethally irradiated amoeba prevents radiation sickness and death. The light portions of non-irradiated centrifuged amoeba progressively lost protective action as the centrifugal force increased from 700 g to about 5,000 g. In earlier publications, Daniels (D2) pointed out that the pattern of migration of the cell components during centrifugation was similar to that of the mitochondria. He subsequently dismissed the participation of mitochondria because his test for mitochondria in the light portion using acid-fuchsin staining seemed negative. However, further investigations would be needed to exclude the presence of mitochondria in the light portion. It is known that mitochondrial fractions are not homogeneous with respect to size, that "heavy" and "light" types exist—the later corresponding to modified mitochondria produced during centrifugation and isolation procedures (G6).

More sensitive tests for mitochondria presence are needed in Daniel's system including direct assay for cytochrome oxidase activity and direct assay for the concentration of organic peroxides as a function of centrifugal force. It is conceivable that the unirradiated mitochondria act as scavengers for radiation produced peroxides while irradiated mitochondria act as a source of these peroxides. It is not certain, however, whether the cytochrome system is involved to any extent in Daniels' amoeba (see p. 189).

The cell nucleus appears to be especially susceptible to the actions of peroxides and strong oxidizing agents (S7). From various lines of evidence, Scheide *et al.* (S7) also points out that the nucleus contains very little anti-oxidants. This would presumably mean that the radiation produced peroxides appear much sooner than in the cytoplasm.

The above considerations have an important bearing on the rate and extent of radiation injury, especially in regard to the

potential efficiency for modifying radiation injury by chemical means after irradiation.

It is of related interest to note that oxygen is a poison to the nuclei of all contemporary cells (S43). In an essay on the origin of life, Sagan (S1) has commented:

. . . the cytological apparatus for dealing with molecular oxygen and oxidizing radiation products is localized in the cytoplasm and is effectively absent from the nucleus. It is, therefore, suggested that the cytoplasm was first developed in primitive times to minimize peroxide contact with the nucleus and to deal with the oxygen evolved from peroxide production . . .

One simple method of defense against peroxides which may have been evolved in primitive organisms, aside from catalase, is simply the resistance to radiation damage by organic and inorganic peroxides provided by the combination of molecular oxygen with the copper component of copper oxidases.

It has been pointed out, p. 29, that the copper levels of primitive organisms are greater than those of higher forms of life. It is conceivable that in the evolutionary scheme, the first organisms to evolve were those in which the predominant oxygen interacting molecules were copper proteins. Later, as additional means of coping with peroxides developed, the more efficient hemoglobin and cytochrome systems took precedence which permitted higher forms of life to develop. However, the unique advantages provided by copper as an oxygen carrier and catalyst has been preserved in the terminal oxidases.

RADIATION CHEMISTRY AND PEROXIDE FORMATION

The formation of organic hydroperoxides and peroxides in aqueous media by ionizing radiation appears to result from the loss of a hydrogen atom from the organic solute by reaction with radiation produced OH radicals. In the presence of molecular oxygen a peroxy radical is formed (A6, H4, S14, W2):



In many instances a radical chain process occurs in which the propagation steps can be written as follows:



Obviously, dialkyl peroxides, ROOR' , can be formed also.

An excellent discussion of the chemistry of the alkyl hydroperoxides and relations between reactivity and structure is given by Walling (W2). It suffices to mention here that the hydrogen abstraction step is rate determining and is in part determined by C-H bond strengths which leads to the usual order of reactivity: Tertiary C-H bonds > secondary > primary. The development of radiation-induced oxidation of organic liquids is favored by the presence of a double bond, by enhanced mobility of hydrogen atoms due to the presence of definite functional groups, and by a straight chain structure as compared to branched and cyclic molecules, particularly aromatic (B1).

Scholes and Weiss (S14) stress the point that there is really no fundamental difference between direct and indirect effects of radiation because an $\text{R}\cdot$ radical can be produced by equation (IV.8) or by direct effect on RH:



Radiation Yields

In Table IV.2 are summarized the estimated yields of hydrogen peroxides and organic peroxides following irradiation of lipids, other organic compounds, and the whole animal itself. It is apparent from the data that the concentration of the solute plays a very important role as shown from the G values for peroxide formation in substances of biological interest.

The "G" value is defined as the number of molecules or ions reacted or altered per 100 ev of absorbed energy:

$$G = \frac{\Delta n}{\Delta E} \times 100 \quad (\text{IV.13})$$

where Δn = the number of molecules reacted/unit volume and ΔE = amount of energy in ev absorbed/unit volume.

It is convenient to estimate the value of G from units ex-

TABLE IV.2
ESTIMATED G VALUE YIELDS (MOLECULES) OF HYDROGEN PEROXIDE AND ORGANIC PEROXIDES FOLLOWING IRRADIATION
100 EV
OF ORGANIC COMPOUNDS IN THE PRESENCE AND ABSENCE OF OXYGEN^a

Substance	Concen- tration ^b M	Low LET (X-rays, Gammas)				High LET (Neutrons, Alpha's)				Refer- ences
		O ₂ -Free		Air-Saturated		O ₂ -Free		Air-Saturated		
		H ₂ O ₂	RO ₂ R	H ₂ O ₂	RO ₂ R	H ₂ O ₂	RO ₂ R	H ₂ O ₂	RO ₂ R	
H ₂ O	Pure	0	0	1.2	0	1.3	0	1.3	0	H8
Cl-	0.1	0.50	0	1.2	0	1.3	0	1.3	0	"
RCOOH (Short-Chain)	<0.1	<0.50	0	2.0-3.4	0	1.3	~0.1	1.3	0.2	"
"	Pure	0	0	<0.2	~3	—	0	—	—	T11
RCOOH (Long-Chain)	>1	<0.50	0	—	—	0 to 1.25	—	—	—	"
"	<0.1	<0.50	0	2.0-3.4	—	≤1.25	—	~1.3	~0.5	H9
"	Pure	0	0	≤0.8	~3	—	0	—	—	"
Saturated Fats	>1	<0.50	0	—	—	—	—	—	—	"
"	<0.1	<0.50	0	2.0-3.4	—	≤1.25	—	~1.3	~0.5	"
"	Pure	0	0	≤0.4	≤3	—	0	—	—	"
Unsaturated Fats	>1	<0.50	0	—	—	—	—	—	—	"
"	<0.1	<0.50	0	2.0-3.4	—	≤1.25	—	~1.3	—	"
"	Pure	0	0	≤0.4	>3	—	0	—	—	"
"	>1	<0.50	0	—	—	—	—	—	>0.5	"
Whole Mouse	—	—	—	—	250	—	—	—	>0.5	"
Nucleic Acids	0.1% W/v	0	0	1.5	1.0	≤1.25	—	—	>0.5	H14
Steroids	Pure	0	0	—	1.2	—	0	—	—	S14
Amino Acids (Tertiary	Pure	<0.50	—	—	1.5	—	0	—	—	"
Carbon Atom)	Pure	—	—	—	—	—	0	—	—	"

^a The estimated G values given in this Table are based on the following assumed yields as given by Hart and Platzman (H8):
Assumed Yields

Assumed Yields	
γ-rays	α-rays
g (H)	3.0
g (OH)	0.5
g (H ₂)	0.5
g H ₂ O ₂	1.45
g (O ₂)	1.25
	1.10

^b In other than the pure liquids, the substances are assumed to be in aqueous solution.

pressed in micromoles per liter, and radiation dosage from Co^{60} γ -rays in rads. Therefore, for aqueous solution we have that:

$$1 \text{ rad} = 6.24 \times 10^{16} \text{ ev/liter}$$

From Avogadro's number, namely, that 1 mole = 6.02×10^{23} molecules, the following expression for G is obtained for a given molecule or ion:

$$G(\text{molecules}/100 \text{ ev}) = \frac{\text{Micromoles molecules reacted/liter} \times 0.965 \times 10^3}{\text{Radiation dose (rads)}} \quad (\text{IV.14})$$

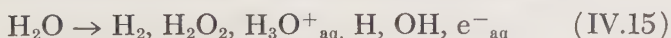
It is easily seen that a G value of unity corresponds approximately to the reaction, alteration, or production of one micromole per liter. See the excellent article by Hart and Platzman (H8) involving G and radiation dosimetry in aqueous systems.

Hydrated Electrons

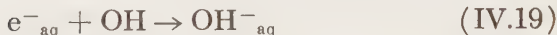
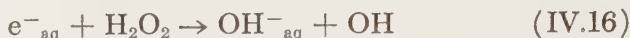
The existence of hydrated electrons, e^-_{aq} , in radiated water has been postulated to explain the production of two transient reducing species produced in the radiolysis of aqueous solutions (H10, M14). One of the reducing species is H and the other carries a unit negative charge. Since the principal reducing species in neutral solutions was postulated to be the hydrated electron, e^-_{aq} , it is of great interest to radiobiology that its existence has been confirmed spectroscopically.

The two principal reducing species in aqueous radiation chemistry are the hydrated electron, e^-_{aq} , and the hydrogen atom, H. However, in aqueous media near the neutral point, the hydrated electrons, instead of hydrogen atoms, dominate as the reducing species. According to Hart and co-workers (H9), not only is the yield of hydrated electrons higher than the H-atom yield, but they react at rates of the order of 100-fold faster than those of the corresponding hydrogen atom reactions if covalent bonds are broken.

Irradiation of water produces the following species:



The hydrated electron is very reactive and its reactions with the other intermediates given in equation (IV.15) are:



These reactions are very rapid with rate constants of $1 - 3 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$. In an oxygenated medium the reaction



probably predominates (H9). Normally O_2^- is quite inert and usually combines with another to form peroxide and O_2 . However, reactions with unsaturated groups might minimize competing reactions (H9). One can also have the equivalent pair of reactions (E. J. Hart, private communication, Oct. 31, 1963):



There is a high reactivity of the hydrated electron with the species represented in equations (IV.16)–(IV.21), but its reactions in neutral and alkaline solutions with methane, methanol, formaldehyde, and the formate ion are unfavorable. In acid solutions the hydrogen atom formed according to equation (IV.17) reacts readily with the latter molecules.

The known electron scavengers, $H_3O^+_{aq}$, H_2O_2 , O_2 , NO , N_2O , and CO_2 have high rate constants with e^-_{aq} as do the organic electron scavengers, $CHCl_3$ and CCl_4 . The organic acids, acetic and formic, react moderately rapidly but not their ions.

Of the chemicals of biological interest, purine, cytosine, thymine, cytidine, and uric acid are very reactive. The amino acids with exception of cystine are unreactive as are the sugars arabinose and ribose. These biological compounds may react by addition of the hydrated electron to form a negative ion as shown in equation (IV.22).

The cupric ion reacts very rapidly with e^-_{aq} . At pH 7 the following reaction with cupric sulfate can occur very rapidly:



The applications of the hydrated electron to radiobiology are just beginning. Adams and Dewey (Ala) have suggested that the reaction of the hydrated electron with certain molecules to form a negative radical-ion can produce radiobiological sensitization (see Chapter XI).

Oxygen Effects

One of the most well-characterized phenomena of radiation biology is the so-called oxygen effect which refers (P2) "to the diminution of many of the changes induced in chemical and biological systems by x and gamma rays when irradiation takes place under conditions of relative oxygen deficiency."

In *dilute oxygen deficient aqueous solutions* the formation of both H_2O_2 and organic peroxy radicals by ionizing radiation of low LET (mean linear energy transfer) such as x-rays and gamma rays is markedly reduced. High LET radiations such as alpha particles, neutrons, protons, and deuterons, however, produce peroxy radicals in amounts nearly independent of the oxygen content of the solution because oxygen forms in the track of high LET particles and fewer radicals escape from the track. Superficially, therefore, it is clear why in certain systems the radiobiological actions of high LET particles are not very much affected by lack of oxygen (S31). When one considers discrete areas of the cell, and especially in mammalian species where discrete radiosensitive structures are in or surrounded by lipids, even high LET particles can be ineffective unless oxygen is present. In the absence of oxygen, highly concentrated solutions, even of lipids, will not produce appreciable amounts of peroxy radicals (H4 and Table IV.1). Therefore, constant or consistent differences of relative biological effectiveness (RBE) between low and high LET radiations for different organisms cannot be expected as, indeed, Zirkle has found (Z2).

Radiation Effects on Copper Oxidases

It is not always an easy matter to measure changes in the

biochemical functioning of metallo-enzymes following irradiation. For obvious reasons, it is best to study the activity of metallo-enzymes following irradiation of the whole animal or of individual tissues. However, the results of irradiation of model systems especially where a simple assay of radiation produced changes can be made, are at the least suggestive.

The irradiation of solutions of ferrous and ferric salts in the presence of complexing or chelating agents has proved instructive. For example, depending on the stability and concentration of the complexing agent, it is possible to obtain not only 100 per cent oxidation of ferrous salts but also 100 per cent reduction of ferric salts under the influence of ionizing radiation (A9). The final ratio of $\text{Fe}^{+3}/\text{Fe}^{+2}$ can be varied simply by suitable adjustment of the type and concentration of the complexing agent. For example, when a complexing agent is present that stabilizes preferentially ferrous iron, such as o-phenanthroline, the reduction of the ferric ion is facilitated, i.e., the oxidation of the ferrous form is retarded. On the other hand, the rate of oxidation of ferrous ion is enhanced when complexing agents favoring the ferric ion are present such as thiocyanate and fluoride ions. Conversely, in the presence of thiocyanide no reduction of the ferric ion under irradiation is observed.

Hemoglobin consists of a protein combined with a porphyrin ring chelated with ferrous iron. When solutions of hemoglobin are irradiated (A6, M27) the first step appears to be an oxidation of iron into the ferric state. Other changes take place, however, which in part are due to the high radiation doses employed. For radiobiology, it is more pertinent to study radiation effects at radiation doses in the region of radiobiological interest.

Hemocyanin: The effects of ionizing radiation on hemocyanin—the copper protein discussed on p. 41—are very interesting and throw much light on the mechanism of action of ionizing radiation on an oxygen-carrying chelate. In fact, the ready availability of hemocyanin in a homogenous solution and the ease by which its oxygen-carrying ability can be measured quantitatively make it a nearly ideal biological model for the study of radiation chemistry of those enzymes and proteins which contain an oxidizable metal as the “active center.”

Previous studies by Svedberg (S46, S47), Barron (B13), and Pickels (P8) on the effects of ionizing radiation on hemocyanin indicated that hemocyanin was highly radiation resistant. Their studies employed radiation doses of from 200,000 r to 600,000 r. The criteria of damage were based on changes in the electrophoretic pattern of the protein (P8, S47) or the actual rupture of the copper-protein bond (B13).

A more reasonable biological criterion of radiation damage is the ability of hemocyanin to combine reversibly with molecular oxygen as measured by the optical absorption maximum at 340 $m\mu$. As was shown by Felsenfeld (F4), the absorption peak at 340 $m\mu$ disappears upon deoxygenation or destruction of oxygen-carrying ability and is about twenty times more intense than the absorption maximum in the visible region. The disappearance of the 340 $m\mu$ maximum is accompanied by loss of the characteristic blue hemocyanin color. In studies on the effect of ionizing radiation or the addition of inorganic or organic peroxides to solutions of serum from *Limulus polyphemus*, the horseshoe crab, the percentage of the oxygen-carrying ability destroyed is conveniently determined on reoxygenated samples of the serum (S23).

In his beautiful studies, Felsenfeld (F4, F5) showed that deoxygenated hemocyanin is much more sensitive to attack than is the oxygenated form and that one equivalent of peroxide per mole of copper is sufficient to destroy most of the oxygen-carrying capacity of the deoxygenated hemocyanin. The peroxide acts by oxidizing the cuprous ion of the deoxygenated hemocyanin to cupric ion. Felsenfeld also found that it is difficult to regenerate the attacked material by the use of reducing agents in the case of hemocyanin from the horseshoe crab but that the hemocyanin obtained from the whelk, *Busycon canaliculatum* can be regenerated. If an excess of peroxide is added in the presence of oxygen, the optical density rapidly rises to that of the original material which regains all of its physiological activity. Possibly a rereduction via equation (IV.5) occurs. Other reducing agents such as potassium ferrocyanide appear to regenerate the *Busycon* hemocyanin.

In investigations of the effect of x-irradiation and gamma radiation from Co^{60} it is found that results similar to that reported

by Felsenfeld (F4) are observed. Some of the experimental findings with *Limulus* hemocyanin are summarized below:

1. Irradiation of oxygenated hemocyanin with as much as 40,000 r cause no change in the absorption maximum as long as the hemocyanin is not deoxygenated. Solutions of oxyhemocyanin stored for several days show no evidence of attack.
2. When solutions of oxyhemocyanin are irradiated with as little as 1,000r and then deoxygenated and reoxygenated measurable oxidation of the copper is detected as shown in Fig. IV.2. (Irradiating deoxygenated hemocyanin produces no changes in the oxygen-carrying ability. This result follows from the fact that no peroxides are produced in the irradiated solutions of deoxygenated hemocyanin). The extent of attack depends on the hemocyanin concentration as reflected in the copper concentration. The copper in the original serum is present at concentration of about $1.2 \times 10^{-3}M$. The G (Cu^{++}) values for initial yield are about 2 whether the value is measured in hemocyanin diluted with phosphate buffer or on the hemocyanin in the original serum. Thus, about 2 micromoles of Cu^{++} are oxidized per 1000 rads.
3. The attack by irradiation is due to peroxides. This is shown by experiments in which H_2O_2 or an organic peroxide is added directly to unirradiated serum. The resulting effects parallel those found in irradiated material, even when the decay of the attack with time is measured. In other experiments it is found that a similar degree of attack results whether the hemocyanin diluted with phosphate buffer is irradiated or whether the buffer itself is irradiated and then added to unirradiated hemocyanin. Addition of catalase to hemocyanin solutions eliminates many of the radiation effects.
4. Because of the catalase-like action of hemocyanin, it is possible to ascertain which action of irradiation is due to hydrogen peroxide and which is due to organic peroxides. This arises from the fact that the rate of decomposition of

H_2O_2 by hemocyanin is directly proportional to the concentration of hemocyanin (F4, G3). Thus, when the serum is diluted twenty-fold with 0.05 M phosphate buffer, the action of irradiation is nearly entirely due to H_2O_2 produced in the buffer while in undiluted serum the attack

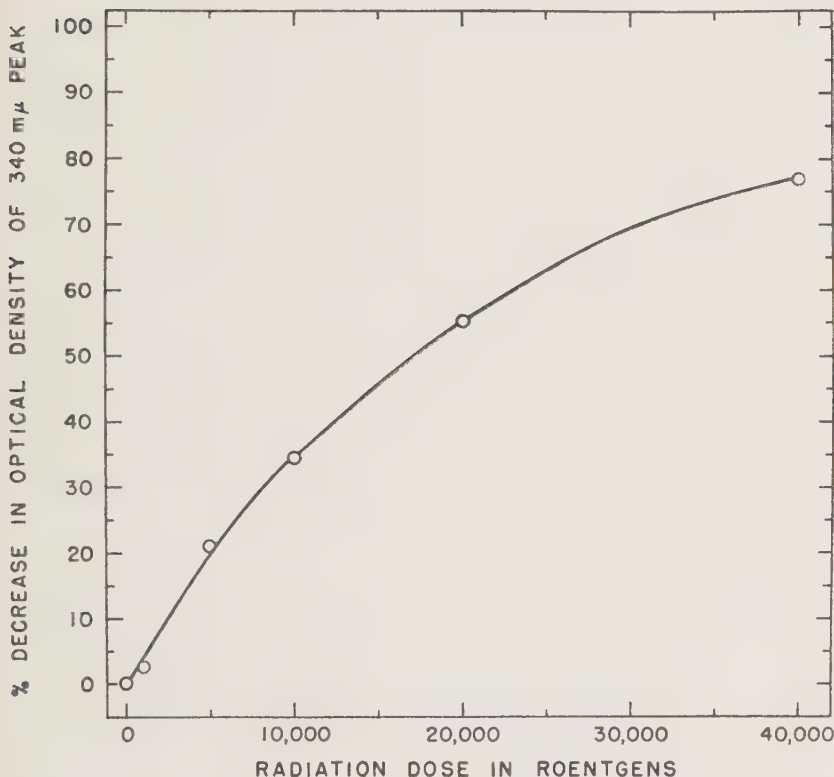


Figure IV.2. Effect of ionizing radiation on hemocyanin (S23). The hemocyanin was present in its natural state in the serum obtained from the horse-shoe crab, *Limulus polyphemus*. The copper concentration in the serum was 1.71×10^{-8} M. The serum was diluted 1:20 with KH_2PO_4 buffer, 0.05M, at $\text{pH} = 7$. The irradiation on the diluted serum $[\text{Cu}] = 8.55 \times 10^{-5}$ M, was carried out at 22°C on air-equilibrated samples at atmospheric pressure with a Co^{60} source at a dose rate of 1,000 r a minute. Measurement of the $340 \text{ m}\mu$ absorption maximum was made on samples which were first deoxygenated by bubbling purified helium through the samples at ten to fifteen minutes after completion of irradiation and then immediately reoxygenated in air.

by irradiation is produced as well by organic peroxides produced from the action of ionizing irradiation on the protein and organic constituents in the serum.

5. Inasmuch as the oxyhemocyanin is radiation resistant it is possible to ascertain the life-time of the radiation produced peroxides and radicals which attack the copper of the hemocyanin. This is done by deoxygenating samples of irradiated hemocyanin at different times after irradiation. The resulting loss in effectiveness of the radiation attack—actually a measure of “after-effect”—is shown by the resulting changes in the optical density at the $340\text{ m}\mu$ absorption maximum. In undiluted serum where the radiation attack involves organic peroxides, the half-time $T_{1/2} = 24$ hours. When the serum is diluted 70 fold with phosphate the radiation attack is nearly entirely due to H_2O_2 and here $T_{1/2} = 70$ hours. At the intermediate dilution of one part serum to four parts buffer the $T_{1/2} = 16$ hours. At the latter dilution the effect of radiation is still nearly entirely due to H_2O_2 because if it is assumed that the decay of the action is due to the destruction of H_2O_2 by the catalase-like action of hemocyanin, one obtains a $T_{1/2} = 70/4 = 17.5$ hours.

Cytochrome Oxidase: In mammalian tissues cytochrome oxidase is one of the most important terminal oxidases. For this reason many investigators have searched for changes in the activity of this enzyme following irradiation but have usually failed to find any significant inhibition (B6, I3, S37, T6 among others.) It is suggested that the failure to find significant changes in cytochrome oxidase activity following irradiation is due to several experimental difficulties, chief among them being a reactivation of the enzyme by the constituents of tissue homogenates. See page 103 for discussion of a somewhat similar problem involving the presumed oxidation of SH-groups *in vivo* after irradiation.

Peroxides have been shown in some cases to inactivate cytochrome oxidase. As little as 10^{-5} mole of benzoyl peroxide can reduce the activity of the enzyme in tissue homogenates. The writer has observed directly a strong and irreversible inactivating

action of H_2O_2 on purified cytochrome oxidase. Yamanaka *et al.* (Y2) have concluded that bacterial sources of cytochrome oxidase (*Pseudomonas aeruginosa*) are destroyed during the oxidation of substrate by the small amount of hydrogen peroxide formed.

Among the reasons it is difficult to demonstrate radiation-induced inhibition of cytochrome oxidase are the following:

1. When assaying for the enzyme, the tissue is homogenized. This releases reducing materials which contact the enzyme and reduce the oxidized copper, thus reactivating the enzyme to a marked degree. Considerable evidence exists showing the ready reduction of Cu(II) to Cu(I) by proteins and cell constituents (F5, F11, K7, K8, N2.)

Frieden (F11) has emphasized the fact that there are several side-chain groups in proteins which rapidly reduce Cu(II) to Cu(I) . Needham (N2) in a similar vein, demonstrated "... that proteins commonly have the power to reduce Cu^{++} to Cu^+ and to do so repeatedly following reoxidation, whether it is bound in nonionic form to the native protein or associated as an ion, after denaturation."

It is possible to minimize the reduction of Cu(II) to Cu(I) in relatively simple systems by either inactivating the reducing action of $-\text{SH}$ groups with an agent such as p-mercuribenzoate (PCMB) or by addition of chelating agents which react strongly with Cu(II) such as EDTA. With some solutions of hemocyanin, these reagents have appeared successful (F5). However, in systems as complicated as homogenized tissue, such an approach has not been successful.

2. Some tissues such as the spleen lose cells a few hours after irradiation. Therefore, it is possible that the very cells in which cytochrome oxidase has been inactivated may not be present.

3. In the assay for cytochrome oxidase, large amounts of cytochrome-c is used as the substrate. It has been found (B10) that cytochrome-c catalyzes the breakdown of unsaturated fatty acid peroxides into inactive products and that during this catalytic process, the cytochrome becomes denatured and has the typical property of catalyzing olefinic oxidation. Thus, a 5×10^{-3} M

suspension of the pure hydroperoxide of *trans-trans* methyl linoleate in a very weak solution of sodium dodecyl sulfate is almost completely and instantaneously destroyed by cytochrome-c at a concentration of 1.6×10^{-4} M and that about a third of the enzyme is denatured (B20). In assay procedures for cytochrome oxidase, concentrations of 10^{-4} M cytochrome are employed. It would be of interest to ascertain the extent to which cytochrome-c destroys radiation-produced peroxides or added chemical peroxides in the analytical procedure for cytochrome oxidase and, in this manner, serves to minimize the degree of inactivation of the enzyme during the assay.

In the absence of stabilizing agents, the most likely systems for demonstrating inactivation of cytochrome oxidase would be purified samples of the enzyme or isolated mitochondria where the presence of reducing agents would be at a minimum. In this connection the experiments of Yost *et al.* (Y3) are of interest. They found that ultraviolet irradiation at 2600-2700 Å will produce nearly complete inactivation of the enzyme in intact cell particulates isolated from rat liver as measured by manometric procedures.

Yost and Robson repeated their studies with ionizing radiation from a Co^{60} source. They suspended the mitochondria in a sucrose-EDTA solution in preparation for cytochrome oxidase assay.

Yost and Robson (Y4) isolated the liver from laboratory rats and washed it free of blood with cold 0.85% KCl. The liver was pressed through a bronze screen to remove connective tissue. The resulting mash was suspended in 50 ml of cold 8.5 per cent sucrose containing 0.005 M disodium EDTA and homogenized. The mitochondria were separated by differential centrifugation (S12). The mitochondria were suspended in 2.5 ml of sucrose-EDTA per gram of original liver. For irradiation, a sample of the suspension was diluted 1 in 20 with distilled water.

The results of irradiation of the mitochondria varied considerably from rat to rat and age of the suspension. Nevertheless, the general trend of the radiation effects can be seen from Table IV.3.

TABLE IV.3

INACTIVATION OF CYTOCHROME OXIDASE FOLLOWING GAMMA IRRADIATION OF MITOCHONDRIA ISOLATED FROM RAT LIVERS^a

<i>Radiation Dose (r)</i>	<i>No. of Runs</i>	<i>Per Cent Inactivation</i>
2,500	2	1.8 $\pm$ 4.1
4,000	8	4.8 $\pm$ 2.4
5,000	13	9.4 $\pm$ 2.9
10,000	6	10.5 $\pm$ 2.2
12,500	8	16.7 $\pm$ 2.4
15,000	14	17.4 $\pm$ 2.9
20,000	19	29.9 $\pm$ 2.4
30,000	5	41.3 $\pm$ 3.5
40,000	6	49.1 $\pm$ 2.8

^a Data taken from ref. Y3. See text p.88 for description of experiment and discussion of results.

The results reported in Table IV.3 are considered minimum values since the degree of reactivation of the enzyme is not known. While EDTA acts as a stabilizing agent, if it can contact the enzymatic copper, it is also known to decompose peroxides as is discussed later in this chapter. It is also known that EDTA inhibits lipid peroxide formation in rat liver homogenates and rat liver mitochondria (B11). Consequently it is necessary to consider the fact that two opposing factors are operating—one is the stabilizing action of EDTA on Cu(II) and the other is the tendency of EDTA to destroy lipid peroxides, thus minimizing the oxidation of Cu(I) to Cu(II).

When comparing the liver mitochondria with other tissues, it is well to remember that the liver contains as much as ten times cytochrome oxidase as other tissue cells. The main point from Yost's study is the fact that cytochrome oxidase activity was inhibited to a measurable degree.

According to the Cu(I,II)-Peroxy Theory, the inactivation of cytochrome oxidase should reach a maximum depending on the rate of formation of the peroxides in a given tissue. Even when the amounts of radiation produced peroxides have returned to normal values, the inhibition of the cytochrome oxidase should persist. In the mouse, for example, the peroxide levels following irradiation with 950 r appears to return to normal in about five days (H14). The efficiency of oxidation, as deduced from the $G(\text{Cu}^{++})$ values (S23) for hemocyanin, suggests that the in-

activation would be independent of radiation dose at levels above 500 to 1,000 r.

The above considerations are of interest in connection with the experiments of Sandberg and Doull (S24). In earlier experiments, Doull and co-workers (D19) had dismissed as unimportant the effects of irradiation on cytochrome oxidase because the per cent inactivation observed was relatively small considering the fact that thousands of r were delivered. Subsequently they repeated their experiments with much smaller doses and found that the inactivation of the cytochrome oxidase was about the same as that found with much higher radiation doses. The degrees of inactivation reported are presumably a minimum.

The experiments of Sandberg and Doull (S4) were carried out on both young and old mice. For reasons discussed on page 156 it is postulated that the absolute amount of cytochrome oxidase in very old animals would be less than in the younger animals. Female mice were used (CF_1) and irradiated with single

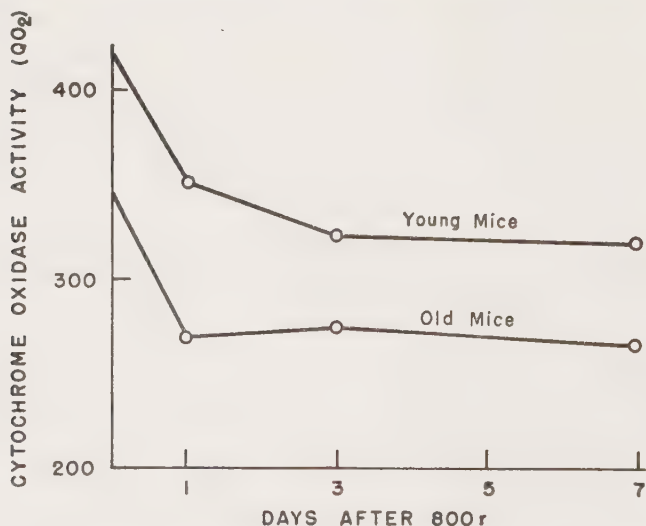


Figure IV.3. Cytochrome oxidase activity in the livers of young and old mice following exposure to 800 r of whole-body x-irradiation. The young mice were CF_1 females, 50 ± 7 days of age while the old mice were ca. 500 days of age. The data are from ref. S4 and are reproduced through courtesy of the authors.

whole-body x-irradiations. At different times after irradiation the mice were sacrificed and the livers assayed for cytochrome oxidase activity by the manometric method of Schneider and Potter (S11). The results of delivering 800r of whole-body irradiation to young and old mice are shown in Figure IV.3. It is hoped that assay methods for cytochrome oxidase will be modified in the future so as to obtain true values for the inhibition of cytochrome oxidase.

IN VIVO PRODUCTION OF PEROXIDES AFTER IRRADIATION

No one has yet been able to quantitatively identify and estimate organic peroxides in *animal tissues*. A long-time worker in the field, J. St. L. Philpot, literally threw his hands up and stated flatly (F2a, p. 55) that: "At present I know of no way of telling whether an animal extract contains peroxide or not." He also stated that he does not know how to estimate peroxide in a mouse and "I have yet to be convinced that anyone else does either." There are, in fact, many good methods for the analysis of peroxides (F2a, H10a) but in relatively simple systems, and even here one is not certain that the methods quantitate more than a limited number of types of peroxides. In the case of animal tissues we are confronted with two as yet unsurmounted difficulties. One is that no one knows how many kinds, chemically speaking, of peroxides are formed—a critical problem because the reactivity of different organic peroxides in a given analysis vary enormously and depend on such factors as the type of solvent, whether oxygen is present or not, hydrolysis times, and trace metal content of the sample. The solvent itself, for example, may produce peroxides through secondary reactions or may induce the decomposition of existing peroxides. Secondly, even if no analytical problems existed there exists the nagging question as to the effects produced by the homogenized tissues on the peroxides.

Some workers have reported that they were unable to detect peroxides in tissues after irradiation or under certain nutritional deficiencies such as Vitamin E. In one case where a synthetic peroxide—ethyl linoleate hydroperoxide—was added to rat tissues, it was found that the intestine though not other tissues destroyed

the peroxide (F2a, p. 90). Who knows what happens to the actual peroxides presumably produced within tissues after irradiation following homogenization and extraction with different solvents?

From the standpoint of an analytical chemist one is appalled at the carelessness of much of the work done on peroxide analysis in tissues. In some cases scarcely little or no controls were conducted in which different types of known organic peroxides were added to tissues before homogenization in order to determine the degree of recovery. It is to be hoped that careful and systematic studies will be undertaken in the future. It may well be that one is dealing with a problem similar to that of cytochrome oxidase assay in that only *in vivo* measurements of a non-destructive nature can be considered dependable.

In spite of the discouraging and disparaging comments made above, there seems to be no doubt that peroxides are formed in tissues. However, when references are made to the formations of peroxides in this book, their concentrations, etc. it must be clearly understood that we are using the frames of references and claims of the respective investigators.

The production of organic peroxy compounds by ionizing radiation in living matter has been amply demonstrated (O1, H14, H15, D16). In the skin (D16) the amounts of peroxide produced corresponded to mean chain lengths of 14 and higher. In the whole mouse an apparent G (ROOH) value of about 250 can be calculated from the analysis of the organic peroxides extracted from the homogenized mouse following an x-ray dose of 950r (Table IV.2).

The accumulation of fatty peroxides in irradiated rats and mice has been reviewed and commented upon by Mal'ts (M3). Of especial interest are his experimental results. He demonstrated that in control rats the peroxide content of the liver was 1.6×10^{-7} mole/g. After irradiation with a dose of 200r, 800r, or 10,000r from Co^{60} gamma-radiation, the peroxide concentration in the liver reached a maximum of about 2×10^{-6} mole/g. According to Mal'ts, the different times at which the individual symptoms of radiation injury appeared in the various rats were matched roughly by the variations in the peroxide levels with time.

It is significant to note that in the formation of radiation-

produced peroxides in fats (H4) and tissues (O1) an induction period is often observed before the characteristic rapid chain reaction takes place. The duration of the induction period is apparently due to the concentrations of antioxidants and inhibitors naturally present in the tissue. According to Hannan and Shepherd (H4) and supported by their experimental data, the autooxidation of a natural fat takes place in two stages: (a) a marked induction period during which the antioxidant is being destroyed, and (b) the characteristic rapid chain reaction.

Another characteristic of the *in vivo* produced peroxides is their long lifetime. In the mouse, for example, overall organic peroxide formation appears to reach a maximum at about one day after irradiation with 950r and gradually drops to the control level five days later (H13). The growth and decay of organic peroxides in individual organs varies considerably. Thus, in the mouse following whole body radiation with 1,000r of x-rays the lipid peroxides appear in the peripheral blood rapidly after the irradiation and continue to increase 36 hours later (O1). In the bone marrow and liver a gradual and moderate increase is observed, but in the spleen a marked increase occurs within three hours which appears to continue even 36 hours later. Oda (O1) claims that the levels of lipid peroxide parallels the damage to the cells.

Different organs possess varying amounts of antioxidants and, therefore, varying abilities to resist or inhibit organic peroxide production. Bernheim and co-workers (B21, O7), for example, have demonstrated that aerobic incubation of brain, liver, and kidney homogenates results in the formation of lipid peroxides while intestinal mucosa or bone marrow do not under the same conditions. Addition of intestinal mucosa extracts to liver homogenates inhibits peroxide formation in the liver. However, 48 hours after exposure (rabbit) to 1400r, marrow homogenates do produce peroxides. The antioxidant activity of rat intestinal mucosa decreases markedly 24 hours postirradiation (O7).

Peroxide Toxicity and Mutagenic Action

From direct measurements of peroxide levels following whole-body irradiation of the mouse (H14), it appears that radiation produces enough peroxide ($0.22 \mu\text{mole/g}$) that, if derived from

linoleic acid, it would kill the mouse (the toxicity, LD_{50} , of auto-oxidized linoleic acid is 0.26μ mole/g). Further evidence for relationships between peroxy toxicity and ionizing radiation is found in the work of Muset and co-workers (M26) who demonstrated a radiomimetic action for lipoxidase.

The poisonous nature of peroxides in fats is exemplified by the well-characterized mildly poisonous nature of rancid fats (B24, D12). The poisonous action can be indirect in that the organic peroxides in the rancid fats destroy essential vitamins, as for example, vitamin A. It has been demonstrated that when all of the essential nutrients are fed separately from the rancid fat to rats that no toxic symptoms develop (K3). It is significant to note that simple addition of antioxidants does not necessarily prevent destruction of essential vitamins by the organic peroxides. The high toxicity of rancid linoleic and cod-liver oil in rats results in weight loss, secondary anemia and leucopenia.

One can view fats in irradiated animals as sites for the production of organic peroxides as well as scavengers for organic peroxides—in other words, as an example of applied radiation chemistry. The production of organic peroxides following irradiation is much greater with unsaturated fats than with saturated fats. On the other hand, saturated fats do produce fair amounts of organic peroxide derivatives when irradiated in air, and these saturated fat peroxides such as palmitic peroxide are poisonous (B24).

Peroxides have been demonstrated to be mutagenic and by inference also carcinogenic (M2, p. 149).

The test method employed the mold *Neurospora*. The spores were suspended in aqueous solutions for thirty minutes and were then thoroughly washed before plating. The spontaneous mutation rate—based on spores which back mutated to adenine independence and thus able to produce colonies on an adenine deficient medium—was determined separately and the observed mutation rates after treatment were suitably corrected.

The effect of hydrogen peroxides and other agents on the induced mutation rate are shown in Table IV.4. It is seen that hydrogen peroxide alone has a very small effect but that in admixture with certain carbonyl compounds, it was an effective

TABLE IV.4
MUTAGENIC ACTION OF PEROXIDES AND
OTHER AGENTS IN *NEUROSPORA*^a

<i>Agent</i>	<i>Induced Mutation Rate $\times 10^7$</i>
Hydrogen peroxide	1.8
Tertiary butyl hydroperoxide	15.4
Hydroxymethyl tertiary butyl peroxide	16.3
Hydrogen peroxide plus acetone	10.4
Hydrogen peroxide plus formaldehyde	40.8
X-rays (dose in r not stated)	21.7
Ultraviolet light (2,537 Å)	43.0
Mustard gas (bischloroethyl methylamine)	4.0
Nitrogen mustard	3.6

^a Taken from data published by Dickey (M2, p. 150).

mutagen. At times the admixtures were assumed to represent "addition compounds" which were not destroyed by catalase. In actual fact, the highly mutant "addition compounds" were organic peroxides which are well known to be resistant to catalase action. Milas and co-workers (M22) have in fact characterized many of the numerous organic peroxides formed when simple aliphatic ketones react with hydrogen peroxide. It has been demonstrated (S24) that ethyl methyl ketone peroxide, for example, is not destroyed by catalase, taking the oxygen-carrying ability of hemocyanin as the test agent.

Vitamin E, Peroxides, and Irradiation

Suggestive evidence for the development of lesions similar to those induced by radiation is provided in studies on the effects of vitamin E (α -tocopherol) deficiency in animals. In their classic studies, Dam and Granados (D1) demonstrated the development of peroxides in the adipose tissues preceding the appearance of a brownish acid-fast pigment. An extensive discussion of the nature of this pigment and its distribution is given by Mason (M13) and Deuel (D12). It is of interest to note that the histopathologic changes in various tissues which accompany vitamin E deficiency and eventually due to excess peroxide formation resemble those induced by irradiation. For example, in the reproductive system of the rat the following degenerative processes are observed (M13): Inhibition of spermatogenesis; diminution of sperm; extensive sloughing of germ cells; the shrunken tubules

are sparsely lined by a vacuolated, fibrous Sertoli syncytium; and the presence of moderate amounts of acid-fast pigment in the Sertoli syncytium.

Other tissues show changes suggestive of those found in irradiated animals. Details are found in the excellent works on vitamin E deficiency by Mason (M13) and Deuel (D12). Among these changes, those in the nervous system are of interest insofar as demyelination and gliosis are found—similar to those observed in copper deficient animals or in animals administered cyanide or azide chronically.

A more direct relationship between vitamin E deficiency and peroxide effects are those reported in connection with the susceptibility of erythrocytes to hemolysis. György and others (G16) have shown that the erythrocytes of vitamin E deficient rats and of newborn rats from mothers on stock diets are readily hemolyzed *in vivo* and *in vitro* by small amounts of hydrogen peroxide. Small amounts of α -tocopherol *in vitro* protect the cells against these effects. Similar findings are reported for monkeys and humans (M13).

Tocopherol levels are low in early postnatal life in humans and tend to increase slowly. During childhood and adolescence the levels become comparable to those of adults—about twice those at birth—and during the latter few decades of life the tocopherols diminish (M13). These changes with age may well have a bearing on the variation of radiation sensitivity with age (page 157).

It might be thought that administration of tocopherol would be beneficial for the treatment of radiation injury (B7, p. 318). However, it is not a simple matter to obtain a suitable distribution of administered tocopherol and, therefore, considerable differences between the effects of high concentrations produced endogenously from those administered may be expected. For example, damage to the male rat's reproductive system as a result of vitamin E deficiency requires the administration of large daily amounts of tocopherol to effect improvement. It is well known that the therapeutic efficiency of tocopherol in a wide variety of disease states produces little or no response unless the tocopherol is administered in relatively high doses over a period of several weeks or months (M13, p. 564).

Chapter V

THEORIES OF RADIOPROTECTION

MANY INVESTIGATORS have suggested in general terms that cytochrome, oxidative phosphorylation, copper, and redox reactions, for example, might be factors in radiation toxicity and chemical protection. The concept of chelation has often been invoked (B6, J2). Many of the proposals put forth have not been useful because they could not be checked experimentally. A good review of existing theories is given in the monograph by Bacq and Alexander (B25) and Thomson (T7).

Caution must be exercised in that undue dependence on the behavior of model systems has led investigators to discard concepts of fundamental validity. While chemical model systems serve as an important basis for studies of mechanism, there are severe limitations associated with the use of model chemical or polymer systems, or even *in vitro* cell suspensions. These systems often require unrealistically high radiation doses of the order of 10^5 r or higher. In addition they lack the full multiplicative properties or metabolic interplay of the whole organism.

CHELATION

Consider some mechanisms involving copper and chelation. In many cases the investigators utilized what they called "copper reagents." However, they did not distinguish between those reagents which preferred cuprous copper and those which preferred cupric copper. Hence, they often reached contradictory conclusions leading them to discard the concept that copper might have been an essential heavy metal concerned in radiation damage. Further, quantitative differences in the strength of the bond between two reagents with preference for the same oxidation state were also disregarded.

The use of chelation as a mechanism in radioprotection has

not appeared fruitful. In one approach (J2) chelation was viewed simply as a mechanism for removal of a trace metal rather than as an efficient means of anchoring the chelating agent to the receptor site. Thus it was hypothesized (J2) that some of the transition metal constituents in the organism were "ejected" from their normal sites by the radiation. The resulting free metal ions then act to poison metal-sensitive enzymes. Consequently, the chelating agent acts to prevent the metal-ion induced inhibition of these enzymes. This hypothesis suffers from several serious shortcomings. For one thing, the concentrations of metal ions that may be released in some manner following irradiation are far less than those associated with the normal chemical toxicity of these metals ($\sim$ one mgm per kgm). Secondly, there exist numerous chelating agents which lack protective action.

There are other reasons for assuming that chelation with extracellular trace metals is not an important mechanism for modification of radiation injury—at least in the mammal. These reasons include the following:

1. Chelating agents with equivalent ability to bind with transition metals should all be active modifying agents, with any differences being due to their metabolic fate. For example, the most stable metabolic chelating agents with consistently high chelating abilities are the polyamino-carboxylic acids such as EDTA. Yet, these compounds as the calcium-sodium salts possess only slight modifying actions (R6, S24) presumably due to their ability to scavenge peroxides. The oft-cited (B5) protective action of EDTA has been ascribed to chelation. However, since high, nearly lethal doses of sodium salt producing hypocalcemia with accompanying convulsions were employed, the reported action is more likely pharmacodynamic (hypoxic). However, it has been proposed (R6) that the protection afforded by sodium-EDTA is ultimately "due to interaction of the mobilized calcium with the adjacent bone-marrow. Calcium could protect the marrow cells by stabilizing their membranes and thereby preventing the loss of intracellular

constituents by a radiation-induced increase in permeability." See Chapter XII for more details.

2. Since penicillamine and cysteine both react by chelation via their sulfhydryl-amino nitrogen groups with copper, iron and other transition elements, it would be expected that if extracellular chelation of trace metals resulted in radioprotection, that the far more metabolically stable penicillamine would be a better protective agent. This is not the case, the radiation modifying action of penicillamine is slight (p. 168).

A more sophisticated approach in which the concept of chelation was applied is found in the work of Bacq and associates (A4, B7). They found that many chelating agents possessed the ability to protect mice from radiation lethality as well as the ability to protect high molecular-weight polymethacrylic acid from decomposition in aqueous solution by x-rays (A4). Unfortunately, they carried the parallel between *in vivo* protection and *in vitro* protection with their synthetic polymer too far. Thus, they suggested that the great activity found with chelating compounds could be due either to the fact that heavy metals such as copper play an important part in the radiation-induced reaction or that the chemical constitution which confers chelating power also has the reactivity necessary for protection. They concluded from their polymer experiments that the latter property was more important. However, an evaluation of their data merely suggests that chelating agents can act by different mechanisms, depending on the system involved. Over reliance on *in vitro* studies led to rejection of their own proposal of a theory of action of cyanide, remarkably similar to some of the concepts suggested in this monograph. In a footnote (B6, p. 313) the following statement was made:

"Bacq and Herve . . . have suggested the possibility that the protector by combining with a radiosensitive enzyme, produces a radioresistant complex. After irradiation this complex slowly dissociates, the protector being eliminated or metabolized (for instance CN^- becomes SCN^- in the liver), and the enzyme be-

coming active again. This idea now seems superfluous, since the CN⁻ ion has proved a good protector for polymers *in vitro* . . .”

Actually, the cyanide ion can protect the polymer, for example, by direct reaction with peroxy radicals and peroxides as discussed in Chapter VII. In the organism, however, this mechanism is probably secondary because of metabolic and biochemical reasons.

From time to time, the chelation of copper has been proposed as a mechanism of radiobiological action. Some of the most recent are described in references A9a, B26, E7, F1a, and K17. Erlendmeyer and co-workers (B26, E7) have shown that heavy metal ions catalyze the oxidation of nucleotides in the presence of hydrogen peroxide. Consequently they suggest that chelating agents such as EDTA by inactivating the metal ions can act as radioprotective agents and thus inhibit the catalytic oxidations of such compounds. Inasmuch as neither EDTA nor penicillamine, which together can chelate all of the biologically important trace metal ions, are nearly devoid of radioprotective action in the intact mammal, the aforementioned catalytic oxidation mechanism is severely limited. It may of course play a role in intracellular regions or indirectly in the case of simple organisms suspended in an aqueous medium.

An interesting role in radiobiological damage has been ascribed to chelation of copper and other metals of variable valence by Anbar (A9a). As one mechanism of protection and sensitization he suggests the following model:

“. . . It has been shown that a certain molecule carrying a copper ion will undergo radiolytic decomposition preferentially to any other organic solute in the system. . . . The selectivity of the induced damage may amount to several orders of magnitude above the statistical probability. Moreover, if macromolecule is affected, it will be the site of the metal ion which will undergo preferential radiolysis. This site may eventually be the active centre of an essential enzyme. . . .

“If the interaction of HO₂ or O₂⁻ radicals with the copper ions is inhibited, or if the copper is removed from its original site by complexants of any kind, these will completely abolish the suggested mechanism of radiolytic damage. Now it is well

known that most, if not all, protecting agents are excellent nucleophils and complexing agents. By blocking complex formation with copper ions they may eliminate the possibility of radiolysing a particular site of a particular molecule, which would otherwise undergo preferential radiolysis."

Further discussion of the suggested sensitization action by copper ions is given in Chapter XI. The presumed role of chelation and copper in the action of sulfhydryl compounds treated in references F1a and K17 are covered in Chapter VIII.

MIXED DISULFIDE HYPOTHESIS

The mixed disulfide hypothesis of Eldjarn and Pihl (E6, P9) is based on the ability of various protective and non-protective thiols to enter into mixed disulfide formation. Cysteine, cystine, and oxidized and reduced glutathione were arbitrarily chosen as prototypes of target molecules since the bulk of the cellular thiol and disulfide groups are parts of cysteine and cystine molecules of peptides and proteins. When a disulfide bond is attacked by the products of ionizing radiation, one of the sulfur atoms is assumed to be oxidized while the other is reduced to an -SH group. Therefore, in 50 per cent of the events the original target sulfur atom will be reconstituted as an SH group, so that mixed disulfide formation is assumed to reduce the chance of irreversible alteration of target molecules. Direct hits from ionization are accounted for by assuming that the additional disulfide bonds serve as electron sources capable of repairing the ionizations by borrowing electrons from a cystine group.

The mixed disulfide mechanism offers no reasonable explanation for the fact that many compounds that do not form mixed disulfides are good protective agents, e.g., dithiocarbamate. It fails to account for the lack of protective effect or sensitization observed with many compounds which do form mixed disulfides. In order for the theory to accommodate various observations, recourse to other mechanisms are invoked. Naturally the actions of many groups of chemical protective agents may range from a specific biochemical interaction to a pharmacodynamic action but the mixed disulfide theory offers no clear-cut *a priori* basis for predicting the action of large groups of compounds.

Many specific criticisms of the mixed disulfide hypothesis have been made (B7, pp. 473-475, T7). An interesting experiment has been performed by Libby *et al.* (L14) to test one aspect of the mixed sulfide hypothesis. They studied the effect of cysteamine on the electron spin resonance spectrum of bovine serum albumin irradiated with Co^{60} gamma rays. Bovine serum albumin was chosen because it contains 17 disulfide groups per molecule but none of these can react with cysteamine because they are sterically inaccessible under isoelectric conditions so that the possibility of protection by disulfide exchange is excluded. The observed protection of the bovine albumin was shown to be due to transfer of energy to the cysteamine rather than by a chemical reaction. The conclusion was reached that:

"Our experiments lead us to question the hypothesis . . . that disulphide exchange plays a part in the protection of animals against radiation by cysteamine, since this mechanism need not be invoked to account for the protection of proteins *in vitro*, whether this occurs directly or by capture of OH radicals formed in water . . ."

THE SULFHYDRYL GROUP

In *in vitro* systems the -SH and -SS groupings, which are important in metabolic processes, appear to be the most vulnerable to the actions of ionizing radiations (B13, M21). Many theories of chemical protection have assumed that the administered drug protects directly or indirectly these sulfur-containing sites so that the action of ionizing radiation is inhibited or deflected. The chemical basis for these theories has assumed that inorganic and organic free radicals, oxygen, and peroxides, for example, produced by the interaction of ionizing radiation with cellular water and tissue constituents are neutralized or dissipated by the protective agent.

In view of the oxygen effect and of the reputed vulnerability of the SH-group, it has been suggested that radiation might affect the levels of free sulfhydryl in tissues. However, no such correlations have been observed. This seems compatible with the fact that the amount of sulfhydryl groups in tissues far exceeds that available for direct oxidation by lethal doses of radiation

(P1). Barron (B13) has asserted, however, that titration of total -SH groups is no indication of the action of ionizing radiations because it is possible that the freely reacting fraction may be present in small amounts compared to the total -SH group. It has been demonstrated that after heavy doses of x-irradiation to the rat, no histochemically detectable primary effect in cytoplasmic protein-bound sulfhydryl or disulfide groups could be found, but in fact definite morphological responses to irradiation occurred without changes in the concentrations of these groups (T4).

It appears that Barron has been correct after all. Ord and Stocken (O2) have reviewed recent work on sulfhydryl groups and found that part of the difficulty in detecting the oxidative effects of irradiation on SH groups is the presence of glutathione reductase. This enzyme is a normal component of isolated cell nuclei so that unless an inhibitor of the enzyme is present such as Zn^{++} , the fall in SH content would not be observed. The response of all cell fractions will presumably depend on the relative availability of glutathione reductase, glutathione, and various compounds involved in phosphorylation. These findings bear a striking analogy to the current problems involved in determining the effects of irradiation on cytochrome oxidase.

Since sulfhydryl and disulfide groups of proteins interact, a temporary formation of oxidized glutathione (GSSG) within the nucleus occurs as the nuclear SH groups decrease after x-irradiation. The addition of GSSG reproduced the oxidative effects of irradiation on nuclear SH groups. The net result of these studies (O2a) was to demonstrate the importance of alterations in the nuclear SH/SS equilibrium in thymus nuclei. It was also pointed out that the biochemical changes associated with the formation of the mitotic apparatus are not completely established, but SH/SS interactions are involved and the process is profoundly affected by exogenous SH compounds.

OXYGEN DEPLETION

The reduction of the oxygen tension of radiosensitive tissues is doubtless the mechanism by which many drugs of pronounced pharmacodynamic activity exert their protective action, e.g., histamine, epinephrine, 5-hydroxytryptamine (M21). It is question-

able whether this mechanism is applicable to many of the thiols, though their pharmacology has not been adequately studied. Oxygen depletion fails to account for such observed facts as that spontaneous oxidation of thiols are poorly correlated with their protective ability (H2) and it is unlikely that spontaneous oxidation of thiols in the usual dosages could induce extensive hypoxia (P9). Whether measurements of oxygen concentration around tissues is an adequate reflection of cellular hypoxia, as Patt pointed out (P5), however, is open to question since it may not reflect tissue-bound oxygen levels.

It has often been found that the protection afforded by cysteine and related compounds could not be explained by simple anoxia, that at least one other mechanism was involved or as Hol-laender (S43) has termed it, a "residual protection action" (p. 133). In experiments on the effects of cysteine and anoxia on the rate of mitosis in lens epithelium, Pirie and Heyningen (P11) clearly demonstrated that the effect of cysteine could be differentiated from that of oxygen deficiency.

In experiments with washed suspensions of *Escherichia coli* B/r Kohen and Gunter (K20) showed that L-cysteine gave protection under different degrees of oxygen tension. They concluded that:

. . . the mode of action of cysteine therefore includes one reaction that can occur independently of oxygen. The independence is both biologic and radiologic; i.e., oxygen is not needed for cysteine to reach the sensitive locus prior to irradiation, nor is it needed at the time of irradiation . . .

It is conceivable that under conditions of low oxygen tension, the hydrated electron might, in part, reactivate cytochrome oxidase via the reaction given in equation (IV.24).

CYTOCHROME SYSTEM

Cohen, Vos, and van Bakkum (C10) have proposed that the radiosensitive site is "localized somewhere in the oxido-reduction chain of the cellular metabolism and that its sensitivity depends on the extent to which it is oxidized. This implies that a high degree of reduction corresponds to radioresistance." They also

locate the site of action at a stage other than at cytochrome oxidase.

However, experimental evidence from radiation chemical studies on cytochrome oxidase and hemocyanin discussed in Chapter IV shows that it is the oxygenated form of protein which is radioresistant. It is interesting to note that Laser (L5) has suggested that protection depends on the stabilization of the reduced stage of a target molecule.

CONCLUDING COMMENTS

In actual fact, the diverse mechanisms by which radiation produces radiobiological effects are matched by the diverse mechanisms by which a protective agent functions. It is necessary, in all cases, to evaluate more specifically, meaning on a more chemical basis, which mechanism of action may predominate in a given case. It is necessary to be able to predict the effects of changes in the molecular structure of a protective agent on its radioprotective properties, to predict the effects of varying dose of protective agent, to predict which drugs can protect against x-rays and not neutrons or against both kinds of radiation. Thus, a working theory must be able to predict, to anticipate, and, above all, be susceptible to experimental testing.

In the following chapter, the Cu(I, II)-Peroxy mechanism is formulated. Subsequently, it is applied in detail to the interpretation and correlation of many experimental observations on the actions of protective and sensitizing agents and to the radiobiological consequences of such physiological and biochemical variables as diet, hibernation, age, and body composition.

Chapter VI

FORMULATIONS OF THE CU (I, II)-PEROXY MECHANISM OF RADIOBIOLOGICAL ACTION

IN THIS CHAPTER, the Cu(I, II)-Peroxy mechanism is formulated in sufficient detail so that in conjunction with the known chemistry of copper and the metabolic fate of drugs the correlative and predictive value of the theory can be tested on experimental data. It cannot be too strongly emphasized that radiation damage to the intact organism involves both the cell nucleus and cytoplasm. However, as mentioned in Chapter I, nearly the entire discussion of the theory is devoted to the cytoplasmic component. Of course, protective agents can and do mitigate in many cases radiation induced nuclear damage. Unfortunately, especially for the goal of effective post-irradiation treatment in particular, the rapidity with which the cell nucleus is damaged means that fully complete protection against radiation is still remote.

Radiation induced oxidation of the cuprous copper of copper oxidase in the cytoplasm simultaneous with the irradiation of the cell nucleus is presumed to set off a chain of events producing biological damage. What these so-called "chain of events" represent or consist of are for the large part still conjectural. It appears that the vital sites or targets are the genetic material in the nucleus and that biochemical injury occurring in any part of the cell becomes manifested or "funneled" into the nucleus. However, one must not confuse the striking histological evidence of chromosome damage or the correlations of nuclear volume and DNA content with radiation sensitivity as proof that the nucleus acts or responds independently of the cytoplasm or that the cytoplasm plays no role or a passive role. Detailed commentaries on these points have been contributed by Bacq and Alexander (B7,

Chapter 9), Kelly (B26a, p. 32 ff), Ord and Stocken (E7a, Vol. I, Chapter 3), and Alper (E7a, Vol. I, Chapter 5).

The field of radiobiology has been replete with "definitive" experiments proving a given theory. However, when repeated under different conditions or with different cells or organisms the results are often contradictory to the expectations of the theory. Such an apparently simple effect as chromosomal breakage long thought to be a primary lesion of irradiation is an end effect seen only a long time after the primary damage (B7, p. 253). The question still remains whether chromosome damage is a cause of cell death or cell death a cause of chromosome damage.

Alper has commented that the DNA molecule, however it may be coiled, stretched, or unwound, is assumed to maintain its integrity in terms of the sequence of its bases. She continues (E7a, Vol. I, p. 408):

"... Any very large variations in the effectiveness per ionization, through the life of the cell, are therefore not to be expected if the mechanism of killing is mainly damage to the DNA. Yet... metaphase eggs of *Habrobracon* are more sensitive than the prophase eggs by the large factor of nearly 22. . . . This implies that for every ionization which is effective in the metaphase egg, 22 are required to kill the prophase egg. If the cells are killed because lethal mutations are inflicted, then at least 21 out of 22 ionizations are ineffective in bringing these about when the egg is irradiated in prophase. It seems very unlikely that any change in the configuration of the chromosomes could account for the marked change in the effectiveness of an ionization; and if physiological conditions can effect "repair" in more than 95% of "damaged" sites, then clearly ionizations in other structures must acquire some relative importance."

In connection with Alper's views those of Bacq and Alexander are also of interest. For example, they point out (B7, p. 268): "... The fact that the sensitivity of the chromosomes to breakage by x-rays can be modified by prior exposure to ionizing radiation and by exposure before and after irradiation to infra-red, also emphasizes that chromosome injuries are not the direct result of the passage of an ionizing particle, but are the result of

a complex interplay of different factors in a living system which reacts to changes brought about in its environment."

The essentials of the Cu(I, II)-Peroxy theory can be summarized as follows: Ionizing radiation damages aerobic organisms by producing excessive amounts of organic peroxides, RO_2R' , and hydroperoxides, RO_2H (B12, B29, D20, F2a, H4, H14, H15, L6, M26, O1, S14) which (1) Induce chemical degradation of nucleic acids and related substances as illustrated by the work of Scholes and Weiss (S14) and Butler (B29), and (2) Promote the oxidation of the cuprous copper component of the four-electron transfer oxidases including cytochrome oxidase, which function via a cyclic shuttling of the copper between the cuprous and cupric states (B18a, M8, M12). Oxidation of the enzymatic Cu(I) to Cu(II) interferes with the ability of these enzymes to interact with molecular oxygen which, perhaps, is harmful to the cell because it reduces the energy available for synthetic processes in the cytoplasm and nucleus.

According to the theory, the goal of chemical treatment is to prevent or minimize the harmful effects of ionizing radiations and can be approached in two principal ways:

I. *Diminution in organic peroxy concentration by:*

A. Indirect means, e.g., hypoxia before irradiation; destruction or inactivation of catalysts promoting peroxy formation such as heavy metals.

B. Direct means, e.g., chemical destruction or scavenging of stable peroxides during or after their production by ionizing radiation.

II. *Stabilization of the cuprous copper component of the essential copper enzymes by:*

A. Indirect means, e.g., maintenance of a sustained concentration of reducing agents in the vicinity of the enzyme.

B. Formation of a dissociable complex or chelate with the cuprous copper component.

It is well to anticipate some important consequences of these protective mechanisms which are discussed in more detail later.

Thus, chemical agents which function by inducing hypoxia will theoretically be effective when administered prior to irradiation but not afterwards. Neither will such drugs be effective against heavy ionizing radiation such as neutrons. However, mechanisms involving peroxy destruction or scavenging such as IB, IIA, and IIB can be expected to be effective against all types of ionizing radiation and to show some effectiveness when administered after irradiation, i.e., against the so-called "after effects" of ionizing radiation. The degree of effectiveness will be dependent upon the dosage of the drug and the schedule of administration. Theoretically, partial reversal of the biological damage would be attained by chemicals or free radicals capable of reducing Cu(II) to Cu(I). Further, drugs which act by mechanism IIB must be capable of forming a chelate with Cu(I). Consequently, changes in molecular structure of a drug which prevent chelation could destroy the protective action.

OXYGEN DEPLETION AND ENHANCEMENT

Any chemical agent which produces oxygen depletion in an organism is assumed to protect only insofar and to the extent that it lowers the oxygen concentration in critical sites. It should also be noted that the protection obtained as a result of oxygen depletion prior to irradiation is usually much more pronounced than that produced by other mechanisms which might be acting concomitantly. As a result, the contributions to protection or even potentiation made by mechanisms other than oxygen depletion may be masked. In certain cases, however, it is possible to distinguish the various actions. For example, *if a chemical agent protects a mammal solely by interference with oxygen transport from the circulating blood then it would be inactive against organisms in suspension in which the oxygen levels can be maintained constant.* Histamine, for example, protects mice but not thymocytes *in vitro* (M21).

It appears that many chemical protective agents act by producing hypoxia in the sense that the dose-reduction factor obtained by the maximum-tolerated quantities of drugs is the same as that effected by the greatest degree of hypoxia an unrefrigerated animal (T7). One must also consider the fact that when a

mammal is deprived of oxygen there is intense stimulation of the sympathetic system which liberates considerable amounts of adrenaline and noradrenaline, substances which themselves possess radioprotective action (B7, p. 289).

There exists a parallelism between the reduction of oxygen tension in the spleen and bone marrow and radioprotection. However, as Thomson (T7) has pointed out, that while a low extracellular oxygen tension implies a reduced intracellular level, the converse is not necessarily true. Some protective agents are known to cause an increase in splenic oxygen tension.

Radioprotection by Oxygen Depletion

There are several ways in which oxygen depletion can be attained. In conventional pharmacology (S40) many types of hypoxia or anoxia are recognized. However, in radiobiology the lumping of the so-called histotoxic anoxia with other types of anoxia is unfortunate. In histotoxic anoxia, the formation of peroxides and free radicals by ionizing radiation is not impaired and may even be enhanced in view of the fact that the intracellular oxygen concentrations are high. Consequently, in order to avoid confusing histotoxic anoxia with the other types of anoxia we will restrict the use of the terms anoxia or hypoxia solely to those conditions in which an actual deficiency of oxygen exists in a given volume of tissue or cell.

The modification of radiation injury from oxygen depletion can be attained, in principle, by the following means:

1. Interference with the transport of oxygen to the cell as in anemia or methemoglobin formation (anemic anoxia) or because of inadequate circulation (stagnant anoxia).
2. Mechanical hindrance in which the free diffusion of oxygen from the alveoli into the blood is prevented (anoxic anoxia).
3. Physical interference with transport of oxygen from the environment to the organism as happens when a bacterial suspension is deaerated or when an animal is given air with reduced oxygen levels to breathe.
4. Chemical or metabolic depletion of oxygen in the medium or circulation. These types of depletion can be induced by simple chemical consumption not involving metabolic processes or by

consumption of oxygen as a result of the metabolism or enzymatic alteration of the added chemical agent. It is apparent that chemical depletion requiring metabolic action would require the administration of the chemical agent for a longer time interval before irradiation than in the case of an agent which acted by direct chemical action as was shown by Stapleton *et al.* (S43).

5. Pharmacological action which produces a lowering of the oxygen concentration in critical tissues or cells. This action can be due to decreased blood flow through particular tissues which may be in turn related to effects on the central nervous system. Whatever the specific mechanism, the important fact here is the lowering of oxygen concentration. Some substances may produce an oxygen deficiency simply by administration of near toxic doses, e.g., induction of a convulsive state in an animal.

Effectiveness of Oxygen Depletion

Protective mechanisms involving oxygen depletion by chemical agents would be expected to be effective under the following conditions:

1. They be administered prior to irradiation or at least be present during irradiation.

2. Against low LET radiation but relatively less effective against high LET radiations. However, in an O_2 -free region differences in relative biological efficiencies between low and high LET radiations insofar as they depend on organic peroxide formation would disappear.

3. The effectiveness of oxygen depleting agents can be expected to increase with increasing concentration of the agent up to a maximum corresponding to a reduction of oxygen concentration to a point where minimization of peroxide production has reached a limit. However, *rate effects* may produce apparent modifications, e.g., rate of depletion of oxygen in mitochondrial lipids may be slower than in the surrounding cytoplasm.

Potentiation of Radiation Toxicity

It is obvious that enhancement of oxygen levels by mechanical, chemical or pharmacological means in tissues and cells, especially those normally low in oxygen, could result in an in-

crease in the production of peroxides. This would be especially noticeable in regions of the cell high in unsaturated lipid content. Very small doses of cyanide can conceivably increase oxygen levels in the mitochondria without a corresponding protection of the copper component of essential enzymes.

PEROXIDE DIMINUTION AND DESTRUCTION

Chemical modification of radiation damage via peroxide decomposition or inhibition of peroxide formation can be attained in several ways. Naturally, not all of these mechanisms can be realized *in vivo* but they offer many interesting possibilities, some of which have been demonstrated—in some cases unwittingly—in radiobiology.

Reduction of Radiation Toxicity

Direct chemical decomposition of organic peroxides: Organic peroxides are decomposed by interaction with many organic agents which act as inhibitors of radical auto-oxidation chains. Amines (p. 69) and phenols are the most common (C16, L14, T10, W2) of such inhibitors. The inhibition reactions appear to involve the following step (W1):



While many substances are theoretically capable of destroying organic peroxides by chemical action, a compound effective *in vivo* in mammals must meet the following requirements: (a) It must be capable of penetrating sites in which peroxides produce radiation injury; and (b) The effective concentration reaching the critical sites must be sufficiently high so that the chemical destruction of the organic peroxides formed by irradiation is appreciable. From the practical standpoint the compound should have a low toxicity and be chemically and metabolically stable, and react rapidly.

Effectiveness of Peroxy Reduction

Chemical agents which can by direct or indirect means reduce the concentration of radiation-produced peroxides would be expected to be effective under the following conditions:

1. They should protect partially even when administered after irradiation though the degree of protection would depend on the time of administration relative to the extent to which the radiation-produced peroxides damaged the critical cellular components.

2. The degree of protection would be a function of both the efficiency of destruction of the peroxides *and* the duration of action of the chemical agent. The latter point is important because of the fact that the peroxide levels seem to increase for several hours after irradiation and remain in appreciable concentrations for several days.

3. Their protective ability should be nearly as great against high LET radiations as against low LET radiations.

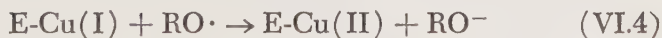
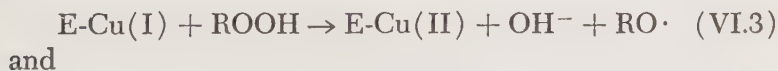
ENZYME AND PROTEIN-BOUND COPPER INTERACTION

Introduction

The ability of copper proteins and enzymes to combine with molecular oxygen resides with the copper component. The deoxygenated forms of these copper proteins and enzymes are especially susceptible to attack by low concentrations of peroxy compounds which destroy the ability of these compounds to react with molecular oxygen.

Oxidative Steps

It is postulated that the copper of the copper oxidase is oxidized by the peroxy compounds produced by ionizing radiation. The oxidation reactions can be depicted as follows:



where E represents the enzyme and Cu(I) or Cu(II) represents fully coordinated copper, i.e., at physiological pH the copper ion is bound to the enzyme and to small molecules in the surrounding medium. Therefore, an added chemical which reacts with the enzyme-bound copper must first displace ions such as hydroxide and chloride.

Let the fraction of Cu(I) oxidized to Cu(II) by ionizing radiation be represented by R_E . By definition:

$$R_E \equiv [Cu(II)/Cu_t]_E \quad (VI.5)$$

where $Cu(II)_E$ represents the concentration of oxidized copper in the protein or enzyme, E, and Cu_t represents the total enzyme-bound copper, that is, $[Cu_t]_E = [Cu(I) + Cu(II)]_E$.

The action of the oxidizing agent, ionizing radiation, on R_E is depicted as:



where R_E° and R_E^i represents R_E° before and after irradiation, respectively, while $R_E^{\bullet}$ represents R_E^i after irradiation with larger doses, i.e., $R_E^{\bullet} > R_E^i$ leading, insofar as the intact untreated organism is concerned, to irreversible oxidation of Cu(I). Since the copper enzyme is insoluble and compartmented within the cell, it is not likely to be reactivated by the reducing agents as occurs during homogenization of the tissue (p. 87).

The values of R_E , by definition, fall between 0 and 1, and the larger the value of $R_E^{\bullet}$ after irradiation the greater is the loss of ability to react with molecular oxygen. Between R_E° and $R_E^{\bullet}$ it is presumed that part of the action of ionizing radiation may be reversible, or, at least that the concentration of peroxy radicals or molecules is kept to low levels as a result of interactions with other cell constituents. It follows that depending on the absolute concentrations of functional copper present, different amounts of radiation will be needed to produce a given value of R_E^i . Speculations as to the implications of this statement for the variation of radiosensitivities between species, and for a given species with age are discussed in Chapter XIII.

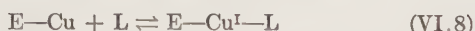
Protective and Sensitizing Mechanism

Protective and sensitizing mechanisms can be expressed most generally as:

$$R_E^p < R_E^i < R_E^{\bullet} \quad (VI.7)$$

Superscripts p and s represent the value of R_E^i when the irradiation is carried out in the presence of protective and sensitizing agents respectively.

For protection by direct interaction with copper

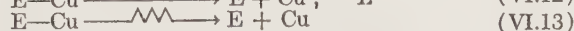
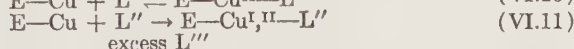


The symbols L , L' , L'' , etc., represent chemical agents added to modify radiation toxicity.

For protection by indirect electron transfer processes¹

$$R_E^p < R_E^i \quad (VI.9)$$

For sensitization by interaction with copper



For sensitization by indirect electron transfer processes

$$R_E^s > R_E^i \quad (VI.14)$$

The reaction represented by equation (VI.13) may result from the breaking of one of the chemical bonds between copper and the protein. Such an action can be produced in copper oxidases by ultraviolet light (A12) or by very high doses ($\sim 300,000$ r) of ionizing radiation as demonstrated by Barron (B13), Svedberg (S46), and Pickels (P8) with hemocyanin.

It is of interest to sketch a general diagram which illustrates one of the requirements of the $Cu(I, II)$ -Peroxy theory insofar as interaction with enzymatic copper is concerned. Beginning with equation VI.5 it follows that the maximum value of R_E will be the same regardless of the presence or absence of a chemical modifying agent since only a finite number of copper atoms is

¹ The term "indirect electron transfer processes" refers to transfer of electrons to or from enzyme-bound copper though no chemical bond is formed between copper and the modifying agent. This occurs, for example, by oxidizing or reducing agents which act by inducing oxidation of $-SH$ groups or reduction of $-S-S$ linkages located elsewhere in the protein. This can result in a transfer of electrons within the protein by the mechanism of H^+ transport suggested by Klotz, *et al.* (K15). Other indirect electron transfer processes are those involving interference along the electron transfer path of the mixed enzymatic catalyst system.

available for oxidation by radiation. Consequently, as a first approximation, an exponential relationship between R_E and radiation dose is assumed to exist as is shown in Figure VI.1. According to the diagram, the effect of a modifying agent is to shift the dose of radiation, D , to a lower or higher value depending on whether the modifying agent protects or sensitizes. It is possible to obtain a relationship between R_E and radiation dose which may more closely approximate experimental findings as, for example, by assuming the value of R_E is formally equivalent to the usual equations (B27) describing oxidation-reduction potentials, namely:

$$E = E_o + C \log \text{Ox/Red} \quad (\text{VI.15})$$

The relationship between R_E and radiation dose shown in the diagram is dependent on many interrelated factors including: (a) the natural anti-oxidant levels in a given tissue; (b) the actual amount of peroxides actually produced as the end result of the

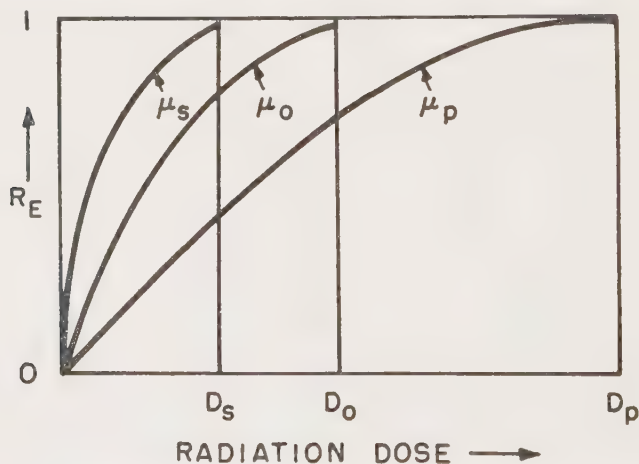


Figure VI.1. Schematic illustration of the degree, R_E , to which the cuprous copper of a copper oxidase is oxidized by ionizing radiation. The subscripts s , o , and p represent the postulated relationship obtained in the presence of a sensitizing agent, absence of a modifying agent, and the presence of a protective agent respectively. The symbol μ represents a rate constant in the presence or absence of modifying agents for the postulated exponential relationship between R_E and a post-irradiation value, R'_E , namely: $R_E = R'_E(1 - e^{-\mu D})$.

interaction of radiation in a given cell volume; (c) the concentration of functional copper available for oxidation; (d) the lipid content and degree of unsaturation of the lipids present at the critical sites.

Agents which modify radiation effects by cuprous ion interaction should, in general, be as efficient against neutrons and protons as against x-rays, should exhibit some activity post-irradiation, and should act independently of oxygen levels.

PHARMACOLOGICAL CONSIDERATIONS

Inasmuch as the Cu(I,II)—Peroxy mechanism is based in part on specific chemical reactions it must obey the law of mass action. Hence, such factors as the concentrations of the drugs relative to the receptor copper atoms or organic peroxides, the relative stability of the drug-copper chelates, and the concentration gradient of the chemical agent must be considered. These are especially critical points when considering the actions of chemical agents which—depending on their dosage—can theoretically protect or sensitize. These chemical factors are directly related to conventional pharmacological factors (A3, W10) such as the transport of the chemical agent, and its chemical alteration through chemical or metabolic processes.

One cannot judge conclusively the effect, or lack of effect, on mechanism of action of a chemical agent on radiation toxicity by results obtained at only a couple of concentrations or at a single dose of radiation or with only one type of radiation. Many chemical agents acting on enzymes produce activation, inhibition, or no response depending on the particular concentration employed relative to the receptor site. Similar biphasic actions of trace metals in pharmacology and on plant growth are well known (A3, C8). Thus, aside from metabolic factors, one cannot necessarily anticipate the action of a chemical agent in radiobiology without knowing the concentrations employed and the effective concentrations reaching the critical intracellular regions no more than one can estimate the final pH of an acid solution following additions of a base without knowledge of the relative volumes, concentrations, and pK's of the reactants.

A serious error made by many investigators in radiobiology

is the assumption that if the maximal tolerable concentration of a drug does not protect against a lethal dose of radiation, neither would a smaller drug dose. In fact—and this was suggested by the Cu(I,II)-Peroxy theory—the protective dose of many drugs may reach an optimum at some intermediate concentrations (Panel I, Fig. VI.2 and p. 126). Of further practical interest is the fact that the optimum protective dose of some radioprotective agents depends on the radiation dose (p. 129).

The same drug may act by two or more mechanisms simultaneously. An agent which may modify radiation toxicity by direct interaction with cytochrome oxidase may also produce hypoxia. Further, many agents which act primarily by affecting the transport of oxygen in a mammal could be ineffective in cell suspensions or bacterial systems or act by another mechanism which would not be apparent or important in the mammal. The Cu(I,II)-Peroxy mechanism furnishes an experimental basis by which various mechanisms of action of chemical agents can be distinguished. For example, if a compound protects animals from lethal doses of radiation by a combination of simple oxygen depletion and cuprous ion interaction, the relative contributions of each mechanism can be estimated by determination of the degree to which the chemical protection is diminished against neutron irradiation as compared with x-rays (p. 178).

Because of the interdependence of the nuclear and cytoplasmic regions of the cell (W11), also noted on page 4, it is especially pertinent to radiobiology to note the intracellular relationships of mitochondria with the nucleus. Novikoff (N4) describes the movements of mitochondria to the nucleus and the intimate contact they make with the nuclear membrane as revealed from cinemaphotography of living tissues. Contact appears to be made at a time when nuclear material appears to be migrating into the cytoplasm. Some authors, on the basis of electron microscopy, consider that the outer layers of the mitochondria and nuclei are continuous. Such continuity has been demonstrated unequivocally in trypanosomes, and from both electron microscopy and cytochemical evidence, the kinetoplast or kine-tonucleus is considered to be a combined nucleus and mitochondrion (N4, p. 391).

The question has often arisen as to what one means by “radio-

sensitivity.” The best answer is that it depends on the end-point chosen and hence cannot be a fixed concept. Thus, in the intact mammal, a common end-point is death which is often expressed as the number of animals surviving after a given number of days. Since radiation damage involves an interaction between the nucleus and cytoplasm of the cell, it can be misleading to restrict the term “radiosensitivity” to the effect on a single site of the cell. Because different cells degenerate at different rates, a given dose of radiation may damage the same proportion of cells in each of two cell populations. Hence, the damage may become apparent more quickly in one tissue than another and therefore, the relative rates of regeneration may then be reflected in the so-called radioresistance of an organ (G5, L2).

We will consider that radiosensitivity refers to death in the intact animal. In the case of tissues we will consider the radiosensitivity to be a measure of the relative amounts of irradiation needed to prevent the tissue from contributing to the survival or well-being of the intact animal. For example, the reproductive processes of the liver can be eliminated without interfering with the usual enzymatic functions of the liver. By the definition employed in this section, the radiosensitivity would be a measure of the amount of radiation necessary to eliminate both the reproductive and enzymatic functions without specifying how much of the cell injury is due to the “reproductive” relative to that involving “energy-transfer.”

In some cells, the biological end-point may be attained mainly because of the reproductive damage. For example, the bone marrow and intestine contains a small number of primitive cells which are absolutely essential to the well-being of the animal (S28). When these are destroyed the animal dies. Here is a case, then, where an insufficient number of cells remain to carry on the biochemical functions, unlike the liver where the cells carry on until they attempt to divide—a rare event compared to the cells of the bone marrow as measured by the mitotic indices.

GRAPHICAL SUMMARY OF RADIOPROTECTIVE AND RADIOSENSITIZING MECHANISMS

In Figure VI.2 is depicted the expected variation of the modifying action of chemical agents toward ionizing radiation as a

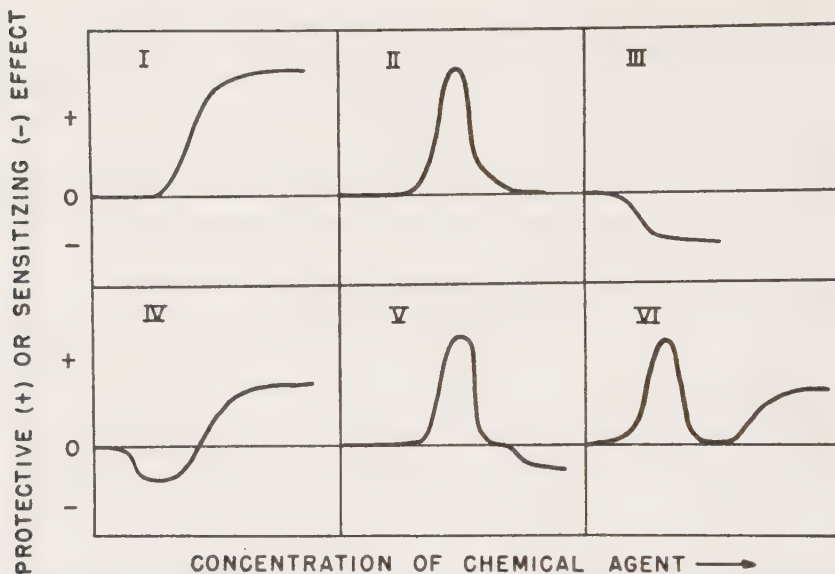


Figure VI.2.—Postulated dependence of the protective (+) and sensitizing (–) action of chemical agents toward ionizing radiation on the concentration of the chemical agents. Curves I to VI represent part of the combinations or mechanisms possible in experimental systems. Discussions of the various mechanisms are given in the text.

- I. O_2 depletion or peroxy scavenging
- II. $Cu^{II}L$ formation (Dissociable)
- III. $Cu^{II}L$ formation (Dissociable) or $Cu^{II}L$ (Non-dissociable)
- IV. $Cu^{II}L$ (Dissociable) followed by O_2 depletion or peroxy scavenging or enhancement of peroxy radical concentration, e.g., increase in intracellular O_2 levels, followed by $Cu^{II}L$ (Dissociable)
- V. $Cu^{II}L$ (Dissociable) followed by $Cu^{II}L$ (Non-dissociable)
- VI. $Cu^{II}L$ (Dissociable) followed by O_2 depletion or peroxy scavenging

function of the concentration of the chemical agent. These curves—which represent only a part of the possible combinations or mechanisms possible—are based on the formulations of the Cu-(I,II)-Peroxy Theory presented in this chapter. It is apparent that practically all agents have the ability to act by more than one mechanism depending on the concentration administered. Consequently, the actual dependence of modifying action with concentration can be expected to become extremely complex in certain experimental systems. Further, a drug which acts by one

mechanism in cell suspension, for example, may act by a different mechanism in another system involving an intact mammalian organism. In some cases, an organism may die before the dosage required to produce sensitizing action may be exhibited, as in Panel V in Figure VI.2. On the other hand, in another organism the test concentration employed may correspond at the outset to that represented in Panel IV.

In the following chapters, a detailed evaluation of experimental data available in the literature is made and the results explained and interpreted in terms of the Cu(I, II)-Peroxy mechanism. It is recognized, however, that caution must be exercised in the utilization of much of the existing experimental data, especially when in most instances the investigators tested only one concentration of an added chemical on the response of an organism. Further, there are unfortunately too many instances where the evaluation of a modifying agent was made in a region of the mortality curve where the experimental results are subject to enormous errors.

Chapter VII

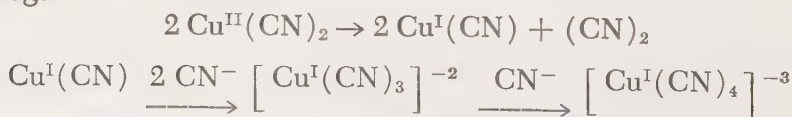
PROTECTION BY CYANIDE AND NITRILES

SEVERAL UNIDENTATE LIGANDS such as cyanide, carbon monoxide, azide, and nitriles react preferentially with copper (I) and are well known inhibitors of copper oxidases. Excellent reviews of the chemical interaction of copper with these compounds are available (B8, C11a, M8, S33). These unidentate ligands have been tested in many radiobiological systems and it has often been found that they possess protective action. However, these same compounds have also been shown to exhibit sensitizing action with some organisms. These apparently contradictory results actually contribute to the understanding of their mechanism of action.

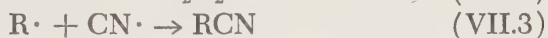
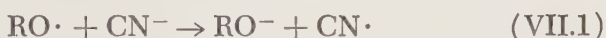
The chemistry of these compounds must be kept in mind, both from the qualitative and quantitative standpoints. For example, while both cyanide and carbon monoxide react preferentially with cuprous copper, the carbon monoxide complex is readily dissociated by oxygen, unlike the cyanide complex. Further, the concentration of these agents employed in the test system must be considered because of expected biphasic actions (p. 117). Disregard of this point has often led to incorrect or misleading conclusions as to the effectiveness of these agents.

CYANIDE CHEMISTRY AND PHARMACOLOGY IN RELATION TO RADIOBIOLOGICAL ACTIVITY

Copper (I) forms very stable complexes with cyanide, CN^- , while cupric cyanide decomposes into cuprous cyanide and cyanogen.

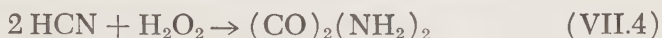


Cyanide ion reacts with peroxy radicals and alkyl radicals. These reactions include the following:



While these reactions could provide extracellular and cytoplasmic protection from damage by free radicals they would not necessarily protect an organism from damage to the genetic structure.

Sollman (S40) reports that cyanide reacts with hydrogen peroxide to form oxamide according to the reaction:



Peroxide has been used as an oral antidote for cyanide poisoning (S40). Hydroxocobalamin which reacts readily with cyanide to form cyanocobalamin (Vitamin B₁₂) is a good antidote (S38a).

The radiobiological actions of nitriles are actually those of cyanide. Therefore, their behavior depends largely on whether a given nitrile yields free cyanide ion during metabolism. The nitriles cannot act as a radiobiological modifying agent by direct interaction with copper because the nitrile complexes of Cu(I), unlike those of cyanide, are decomposed by water and metallic salts.

According to the Cu(I, II)-Peroxy theory, the radiobiological action of cyanide can run the gamut from protection to sensitization depending on the concentrations employed. Hence, experiments with cyanide or other agents acting by similar mechanisms should utilize a wide range of concentrations. From a chemical standpoint, that is, the law of mass action, it is anticipated that too low a dose of cyanide would offer no protection while too high can also be ineffective so that the optimum dose would lie in between as depicted in Figure VI.2, Panel II.

In the case of organisms or cells in suspension, the action of cyanide could more likely produce sensitization via equation VI.11 because the concentrations of cyanide employed relative to the receptor sites have been several orders of magnitude higher than in the case of mammals. In one investigation, for example,

a concentration of nearly 0.1 M KCN was employed (W1). However, the usual range of cyanide used in suspensions of organisms and cells has been of the order of 10^{-3} M.

One of the principal detoxication mechanisms for cyanide (W10) in the mammal is its conversion by the enzyme rhodanese to thiocyanate via a reaction which utilizes endogenous thiosulfate as shown by the following reaction:



In the very small doses cyanide can be metabolized by processes other than thiocyanate formation since it appears there exists a small metabolically active pool of cyanide in the body (W10).

The ability of a mammal to detoxify cyanide is related to the levels of the enzyme rhodanese present in the tissues. It is interesting to note that the frog and rat contain the highest levels of rhodanese of the common mammalian species (W10).

Another pathway of detoxication of cyanide involves a reaction between cystine and cyanide to form 2-iminothiazolidine-4-carboxylic acid (ITCA). Daily subcutaneous injection of KCN into rats results in the excretion into urine of ITCA corresponding to 15% of the dose of KCN (W10). The ITCA has also been detected in the saliva and urine of humans exposed to cyanide. Further discussion of ITCA is given in Chapter XII in connection with its post-irradiation protective properties.

There exists a reverse pathway for converting thiocyanate to cyanide by means of the enzyme thiocyanate oxidase (W10). This enzyme is found only in the erythrocyte and has been detected in man, dog, rabbit, and rat. This indicates that minute amounts of cyanide are essential in metabolism (S38a) and it may explain some of the observed toxic effects of thiocyanate which resemble sub-acute cyanide poisoning.

Two mechanisms of direct copper interaction are possible, namely one of protection, as depicted by equation VI.8, and one of sensitization via the reaction shown in equation VI.11. It is unlikely that the receptor copper atoms would be removed from the protein as depicted in equation VI.12 because this would require a sustained concentration of > 0.1 M in cyanide.

In mammals it is difficult for sensitization via reaction VI.11

to occur because the toxicity of cyanide on the sensitive respiratory centers may preclude the use of a dose high enough to maintain a nondissociable complex ion. Thus, in the experiments of Bacq (B3) the protective dose of sodium cyanide corresponded to a maximum of 10^{-4} M if uniformly distributed. However, taking into account the metabolic fate and excretion and distribution it is unlikely that the actual concentration of cyanide in the vicinity of an enzyme such as cytochrome oxidase following a dose of 5 mg/kg exceeds 10^{-6} M. It has been found that partial inhibition of cytochrome oxidase in liver homogenates by cyanide occurs in this concentration range (A2). More to the point, however, injection of 5 mg/kg of NaCN to mice produces 40 per cent inhibition of liver cytochrome oxidase (S22).

From conventional considerations it is difficult to account for the radioprotective actions of cyanide. Van Bekkum and Zaalberg (B19), for example, pointed out that the "explanation for the *in vivo* protection by cyanide is not evident because cyanide is known to inhibit the respiratory metabolism of the cells, which would presumably result in *increased* oxygen levels inside the cells." Since the oxygen is not utilized, the production of peroxides may be higher than normal—a situation which may enhance genetic damage in the nucleus. We will not examine the complicated effects of radiation on the genetic structure in relation to cyanide, but an extensive discussion can be found in the articles by Kihlman *et al.* (K6) and Sobels (S39).

The immediate cause of death from cyanide in higher animals is paralysis of the respiratory center (S40). Consequently the intrinsic tolerance of the animal to the full biochemical or radiobiological action of cyanide may not be realized. However, the high doses of cyanide employed for most radioprotective purposes in mammals appear to produce hypoxia in the spleen and bone-marrow (M21, 1961). It is claimed that the hypoxia explains the protection observed in the mouse against a lethal dose of x-rays. While other mechanisms of protection are not precluded, there are several reasons to question whether hypoxia in the case of the mouse is of paramount importance and, in fact, whether it may be a redundant side reaction.

The data presented by van der Meer *et al.* (M21) showed

that the average maximal reduction in oxygen tension in their mice was the same for animals receiving about 5 mg/kg of KCN or 3.75 mg/kg of KCN, namely 82 and 84 per cent respectively. Yet the 30-day survival dropped from 81 per cent to only 30 per cent with the lower dose of KCN. As a result, the investigators claimed that the significant factor was the longer duration of the hypoxia in the mice receiving the higher doses of KCN, namely, 30 minutes compared to 21 minutes. It seems unlikely that the duration of post-irradiation hypoxia is a factor in these studies. Mammals which have been subjected to hypoxia post-irradiation are not protected and neither does the introduction of oxygen immediately post-irradiation affect the lethal dose.

It was also observed (M21) that a reduction of 72 per cent in the splenic oxygen tension with an average duration of 15 minutes gave nearly no protection (8 per cent survival). Yet in the case of histamine, a dose which produced a reduction of only 46 per cent in oxygen tension with a duration of 13 minutes gave a survival of 46 per cent in the same mice by the same group of investigators (M21, 1959). Further, in experiments reported in this Chapter it is shown that mice can be protected by as little as 1 mg/kg of cyanide—a dose which would have relatively much less effect than those employed by van der Meer *et al.* Neither does the hypoxic mechanism explain the decrease of radioprotection with increasing dose of cyanide described below. Since the mice employed by van der Meer *et al.* could tolerate up to 10 mg/kg of KCN it would have been interesting to have determined whether a decrease in radioprotection took place between 5 and 10 mg/kg of KCN.

It is possible that hypoxia plays a role in the action of cyanide but it is more likely that it would still protect even if no hypoxia was produced as is the case with some of the sulfhydryl protective agents.

It is postulated that an important radiobiological mechanism of action of cyanide (and of nitriles) involves the chelation and hence protection of Cu(I) of copper oxidases against chemical attack by peroxides. If the concentration of CN^- is too high, the resultant overstabilization of the cuprous copper could produce prolonged inhibition of the enzyme and produce a radiomimetic

action, i.e., sensitization. If the cyanide level could be reduced or actually removed *concomitantly with the peroxy compounds* after the irradiation, then the net result should amount to a protective action because of the shielding of the cuprous copper during the period of irradiation.

Theoretically, cyanide could potentiate radiosensitivity in mammals at very *low* doses as well. The mechanism postulated is as follows: the dose of cyanide is assumed to be large enough to interfere with oxygen utilization and hence increase the oxygen levels within the mitochondria. Following irradiation a higher than normal concentration of peroxides would be formed. The copper-bound cyanide would be lost as a result of dissociation—a reaction favored by very low concentrations of cyanide. The resulting curve of radiation modification could then be expected to resemble that shown in Panel III of Figure VI.2 at low concentrations followed by the curve shown in Panel II.

RADIOBIOLOGICAL EXPERIMENTS WITH CYANIDE

Protection of Mammalian Organisms

When injected before irradiation in high but non-lethal doses, sodium or potassium cyanide is found to protect mice (B6, B7, B3). However, little or no protection had been reported for the rat (D21, Y1) or frog (P2). The frequently cited results of Dowdy *et al.* (D21) with rats were obtained using a single dose (3 mg/kg) of sodium cyanide administered three minutes before irradiation. The dose was high enough to cause collapse in the rats but was “definitely sublethal, and recovery was usually complete in twenty minutes.” None of the treated rats survived an x-ray dose of 800r. Yakovlev and Ivanov (Y1) investigated doses of NaCN of from 1.4 to 4.7 mg/kg in rats and found only 33 per cent survival in those given 2.8 - 3.8 mg/kg of sodium cyanide.

The Cu(I, II)-Peroxy mechanism suggests that the dose of cyanide required to achieve protection in irradiated animals would be sharply defined, and, if not limited by chemical toxicity of cyanide, it should be possible to demonstrate protection in the rat as well as the mouse using the proper dosage. Accordingly, experiments were carried out comparing survival in rats and mice

given a wide range of doses of sodium cyanide prior to x-irradiation (S22, S24).

The animals were given sodium cyanide solutions intraperitoneally 3-6 minutes before irradiation by x-rays. Preliminary toxicity data on sodium cyanide given intraperitoneally were obtained. For mice, the 30 day LD_{50} was 6.3 mg/kg and the maximum safe dose was 5.5 mg/kg. For rats, the 30 day LD_{50} was 4.0 mg/kg and the maximum safe dose was 3.0 mg/kg. The irradiation experiments were carried out at two levels of x-irradiation for each species. Female CF_1 mice, ANL bred, about 70 days old were employed. Their weights averaged 25 ± 1 gram. They received x-rays at a dose rate of 38r/min. The rats were Sprague-Dawley females, ages 7 to 8 months, and their average weights were about 270 grams and fell in the range of 260 to 285 grams. They received x-rays at a rate of 21.4 r/min.

The results, parts of which are shown in Figure VII.1, have borne out the expectations of the $Cu(I, II)$ -Peroxy mechanism. Significant protection was obtained in both rats and mice. In the case of mice given 750 r the degree of protection increases with increasing dose of cyanide, beginning at about 2 mg/kg. However, in the rats given 850r cyanide exerts no appreciable protec-

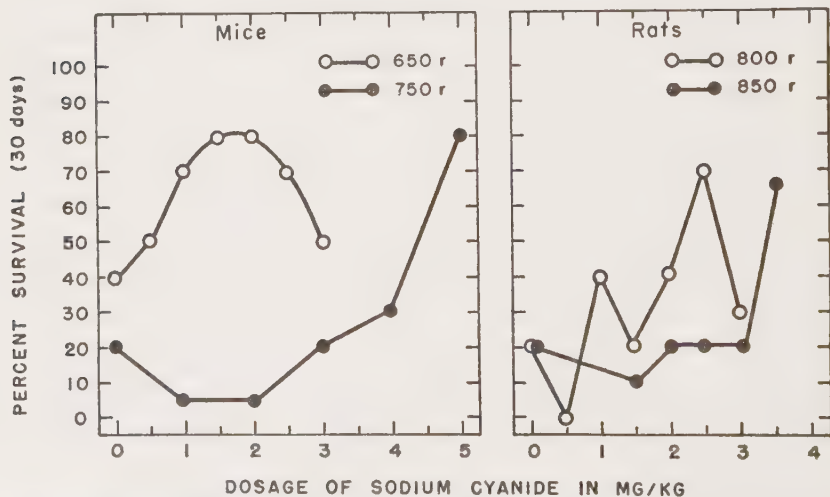


Figure VII.1. Effects of varying doses of sodium cyanide administered prior to X-irradiation on protection of mice and rats (S22, S24).

tion until a dose of about 3 mg/kg is reached where a sharp rise in protection ensues. Two separate experiments were made to check this rise. In one, 50 per cent survival was observed and in the other, 67 per cent. Considering the abrupt response these results show good agreement.

Experiments at a lower level of irradiation showed that for both the mouse and the rat the minimum amount of cyanide required to bring about significant protection was less than that needed for higher irradiation doses. The lower cyanide doses were about 1.0-2.0 mg/kg for mice given 650r and 2.0-2.5 mg/kg for rats given 800r. The shift in the dose of cyanide needed to provide maximum protection is also reflected in an approximately equivalent shift in the concentration of cyanide required for the protective action to begin its rise. It must be emphasized that quantitative reproduction of these data in the vicinity of the LD_{50} is not observed. Hence, one obtains the striking result that *a dose of cyanide which protects against a high dose of radiation can be completely ineffective against a lower dose.* It is concluded that for any radioprotective drug which acts by the same mechanism as cyanide, a dose which protects against an LD_{90} dose may be completely ineffective against an LD_{50} dose of radiation!

The observation that the radioprotective dose of NaCN increases with larger doses of irradiation is noteworthy both from theoretical and practical points of view. Since cellular permeability generally increases with increasing irradiation dose (B7) the explanation probably lies elsewhere. It is tempting to speculate that a contributing factor to the cyanide-radiation dose relations arises from the fact that cyanide ion reacts with peroxy radicals and hydrogen peroxide. Consequently, since the concentration of peroxy radicals produced *in vivo* presumably increases with increasing radiation dose, the dose of NaCN needed to reach critical intracellular sites would necessarily increase. An elucidation of the mechanism involved would entail a study of the degree of protection as a function of the time elapsed between the administration of cyanide and onset of irradiation. It is also conceivable that these observations reflect a radiation induced inhibition of rhodanese (p. 124).

The Cu(I, II)-Peroxy mechanism suggests that the cyanide forms a cuprous cyanide complex with enzymes such as cytochrome oxidase which eventually dissociates. It is suggestive to note that the observed inhibition of the enzyme in mice (p. 50) approximate the dosages needed for protection. Unfortunately no radiation induced inhibition of cytochrome oxidase in mice or rat tissues were demonstrated despite the deliberate addition of a Cu^{++} stabilizing agent, EDTA, to the tissues before homogenization (S24). However, significant levels of inhibition of liver cytochrome oxidase from mice and rats following irradiation have been reported (S4, Y3). The latter data are discussed in some detail in Chapter IV. Some artifacts involved in the assay of cytochrome oxidase have been discussed on pages 87 to 89. It would be worthwhile to be able to measure the inhibition *in vivo* of cytochrome oxidase as a function of time after administration of cyanide and as a function of radiation dose in different tissues.

At very low doses of cyanide, less than 1 mg/kg, a sensitizing action was noted. However, the sensitizing action was significant with respect to the rate of death rather than in terms of 30-day survival (S24). In any event, the sensitizing action is most interesting but because it is relatively small, it must be studied in more detail with larger numbers of animals as a function of radiation dose. In mice receiving an approximate LD_{50} of ionizing radiation, 650r, the sensitizing action was observed at 0.3 mg/kg and reached its maximum effect at 0.5 mg/kg after which it rapidly disappeared.

It is apparent that the reason previous investigators have overlooked the radioprotective action of cyanide in rats is due to the very narrow dose range, less than 0.5 mg/kg, over which protection is obtained. Further, the use of only one dose of cyanide or of irradiation also made it unlikely that protection would be observed. There remain many other factors to be investigated. However, the fact that the degree of protection is easily influenced makes it difficult to obtain reproducible data. It has been pointed out (T7) that the radiobiological actions of cyanide may be influenced by the dose rate at which the radiation is delivered, and the time elapsed between administration

of the cyanide and the irradiation. Thus, it is stated (T7) that even an interval of two minutes between injection and irradiation with a dose of 700r to mice is too long for cyanide to provide significant protection. Yet in other experiments (S22) significant protection in mice is observed even when cyanide was given six minutes before irradiation.

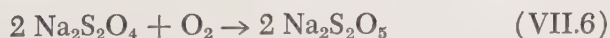
Protection of Non-Mammalian Organisms

The ability of cyanide to protect organisms during irradiation by presumably combining with the copper component of essential enzymes is suggested by the interesting results of Laser (L5) quoted below:

In the case of *Sarcina lutes* it has been possible to abolish almost completely the "oxygen-effect" at doses up to 26,000r, if cell respiration was inhibited by respiratory poisons during the irradiation. Thus, after removal of the poison, the cells behaved as if they had been irradiated in nitrogen, when judged by the degree of growth inhibition and subsequent rate of recovery. The effective inhibitors were carbon monoxide, potassium cyanide, hydroxylamine and sodium azide . . .

Note that all of the inhibitors employed react with cytochrome oxidase via the copper (I) component. Presumably the peroxy radicals also are washed out (p. 127) along with the cyanide in Laser's experiments.

The careful experiments of Stapleton, Billen, and Hollaender (S43) involving the irradiation of suspensions of *E. coli* in the presence of various chemical agents plus cyanide are suggestive of Cu(I) stabilization. In these and other experiments Hollaender *et al.* B28, H11, S43) showed that two groups of chemical agents protected bacteria because of oxygen removal. One group reduced oxygen levels as a result of metabolic processes in which the agents were utilized as oxidizable substrates while another group, typified by sodium hydrosulfite, $\text{Na}_2\text{S}_2\text{O}_4$, reduced oxygen levels by chemical interaction with dissolved oxygen:



The end result, namely protection, was the same. However,

those compounds which removed oxygen as a result of enzymatic action required preincubation in order to protect while those compounds which removed oxygen independently of enzymatic action required no preincubation period.

A plot of the surviving fraction (plate counts) of *E. coli* versus the molar concentration of $\text{Na}_2\text{S}_2\text{O}_4$ or of O_2 concentration is shown in Figure VII.2 and gives the same kind of curve depicted in Panel I of Figure VI.2.

A significant step introduced into this experiment involved the addition of cyanide to the suspension in order to ascertain whether the action of the oxygen removal agents could be modified. The amount of cyanide added (2×10^{-3} M) was in itself nearly inactive but was reported to have increased slightly the radiosensitivity of the cells. Inasmuch as cyanide is known to prevent the metabolism of those agents which remove oxygen

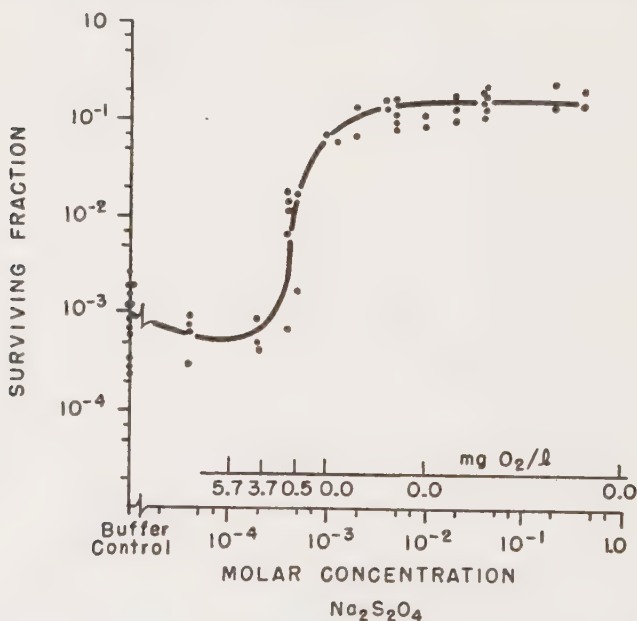


Figure VII.2. Survival of *E. coli* B/r in different concentrations of sodium hydrosulfite exposed to 60,000 of x-rays and kept at 2°C during exposure. The data are based on plate counts and the illustration is adapted from that given by Hollaender and Stapleton (H11).

enzymatically it was expected that the protective action of these substances would be abolished. This is what happened. The protective action of formate, succinate, pyruvate, and serine was abolished by the presence of cyanide. However, those agents such as sodium hydrosulfite which act to remove oxygen by chemical action retained their protective action. The explanation here (genetic effects were not determined) is straightforward—namely that the production of organic peroxides by irradiation is drastically reduced in an oxygen deficient medium and, therefore, the protection of the enzymatically-bound cuprous copper by cyanide is superfluous. However, for a more definitive evaluation of the mechanisms, the concentration of cyanide would have to be varied.

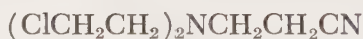
That fraction of the protection action of drugs which are postulated to function via direct combination with enzymatically-bound cuprous copper would not be expected to be affected by cyanide in the experiments described here. Therefore, it should be possible to determine the relative contribution to protection afforded by oxygen depletion versus direct combination with receptor copper. The apparently anomalous results cited by Hollaender *et al.* (H11, S43) with cysteine can be interpreted by the Cu(I, II)-Peroxy theory. Thus they found that cysteine and other sulfhydryl compounds removed oxygen and were able to protect *E. coli* without incubation. As just mentioned, this mechanism of action will not be affected by cyanide. However, the protective ability was only partly accounted for by oxygen removal which led the investigators to speak of a “residual protection action.” This “residual action” is here presumed to be due to combination with the cuprous copper receptor site. It is of interest, therefore, to note that the overall protective action of cysteine was reduced only in half by cyanide. Hence, it can be concluded that in this system half of the protective action of cysteine is due to simple oxygen removal and half to combination with the cuprous copper receptor. This conclusion has a bearing on the degree of protection afforded by various agents including cysteine against x-rays as compared with neutrons—a topic discussed in Chapter XII.

RADIOBIOLOGICAL EXPERIMENTS WITH NITRILES

Further insights into the actions of cyanide are provided by the radiobiological actions of nitriles. The radiobiological behavior of nitriles is assumed to depend on their ability to release cyanide on metabolism. Consequently, to a first approximation, it would be anticipated that only those nitriles whose toxicity on a molar basis is comparable to cyanide would be protective. On the other hand, nitriles of low chemical toxicity should be ineffective against radiation toxicity.

More than twenty nitriles tested by the USAF radiation laboratory (D10) were examined. Among these compounds only two had toxicities corresponding to that of cyanide itself. These were hydroxyacetonitrile, HOCH_2CN and malononitrile, $\text{NC-CH}_2\text{-CN}$.

All of the low toxic ($\text{LD}_{50} > 1\text{mM/kg}$) nitriles, regardless of structure, were ineffective. A summary of part of these observations is given in Table VII.1. These data fully support expectations. One compound not mentioned is at least as toxic as cyanide but had no radioprotective action. However, the compound, shown below, is a derivative of nitrogen mustard and it is highly toxic without its nitrile groupings as well.



Propionitrile, β -bis-(2-chlorethylamino)

The fact that low toxicity actually means that little cyanide is made available to the mammal is borne out by more detailed examination of the metabolism of some of these compounds. For example, the low toxicity of acetonitrile is due to the fact that it is rapidly and largely converted to thiocyanate and formic acid (W10). Succinonitrile, unlike malononitrile is, in part at least, converted *in vivo* to succinic acid and ammonia without producing appreciable cyanide.

Aryl cyanides are not expected to possess radioprotective action since they do not produce HCN metabolically. In fact, they are relatively non-toxic and do not have radioprotective effects to any significant degree. Some of the alkylaryl cyanides,

TABLE VII.1

RADIOPROTECTION ACTION OF NITRILES IN MICE AS A FUNCTION OF TOXICITY^a

Nitrile	Formula	Toxicity		Protective Action ^b
		mg/kg	mM/kg	
Cyanide	NaCN	6-9	0.12-0.18	+
Hydroxyaceto-	HOCH ₂ -CN	10-25 ^a	0.18-0.44 ^a	+
Malono-	NC-CH ₂ -CN	25 ^a	0.12-0.38	+
Aceto-	CH ₃ -CN	500	12	0
Succino-	NCCH ₂ CH ₂ -CN	100-200	1.3	0
β-aminopropio-	H ₂ NCH ₂ CH ₂ -CN	>1000	> 14	0
Cyanamide	H ₂ N-CN	200-300	4.8-7.1	0
Bis-(2-cyanoethyl)amine	HN(CH ₂ CH ₂ -CN) ₂	200	1.6	0
Phenyl aceto-aceto nitrile	C ₆ H ₅ CH ₂ COCH ₂ -CN	200-300	1.3-1.9	0

^a The data were taken from the listing given in reference (D18) except for cyanide (S24). The toxicities of the hydroxyacetoneitrile and malononitrile are probably more than reported here because later analyses (private communication) revealed that concentrations were actually less than had been thought. In unpublished work it was found that 8 mg/kg is protective in mice (S24).

^b The tests of protective action are based on sublethal doses of the compounds listed in the table.

however, are highly toxic, as for example, mandelonitrile (6 mg/kg in rabbits) while cyanoacetic acid is relatively non-toxic (200 mg/kg in rabbits) because its cyanide group is biologically and metabolically stable.

Since the radiobiological action of nitriles is essentially that of cyanide, the variation of radioprotective action with drug dosage should be similar. Doull and co-workers (D18) measured the protective action of malononitrile and hydroxyacetoneitrile at four different concentrations. The results are plotted in Figure VII.3 and confirm expectations. In experiments (S24) using smaller increments of malononitrile a more detailed dose-response curve has been obtained which confirms that shown in Figure VII.3. The explanation of the results follows that already given for cyanide.

Experimentally, it is difficult to obtain reproducible results with nitriles because they often undergo chemical changes in solution. Hence, the actual concentration administered may vary from day to day. In view of the dependence of the radiobiological effects of nitriles on dosage, it is obvious that strict chemical calibration of the solutions is needed, e.g., nitrile assay, and free cyanide assay as a minimum. In addition, biological testing of

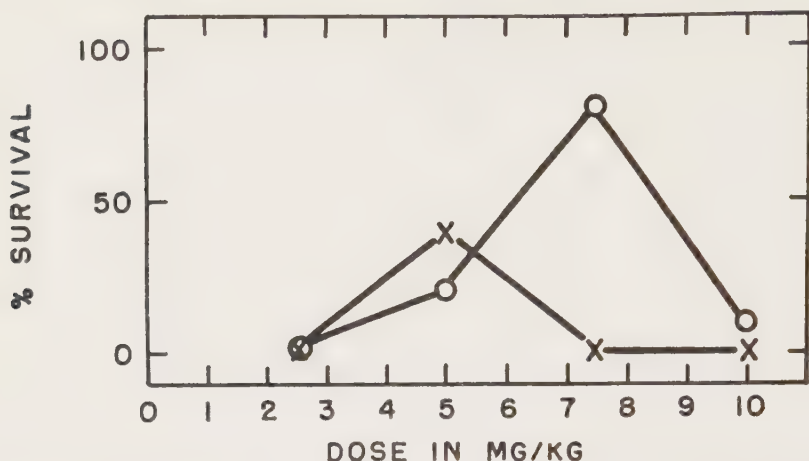


Figure VII.3. Radioprotective action of two nitriles administered prior to irradiation in mice receiving whole body X-irradiation with 800 r as a function of nitrile dosage. The data are taken from reference D18 and represent survivals 30 days post-irradiation. See Table VII.1 for further information.

o—Hydroxyacetone nitrile
x—Malononitrile

the nitrile solutions is needed to insure that no subtle changes in the molecular structure had taken place, as it is sometimes found that the toxicity of the nitrile may change markedly on standing even though no obvious chemical changes had occurred.

Chapter VIII

PROTECTION BY SULFHYDRYL COMPOUNDS

CHELATION AND CU(I) INTERACTION

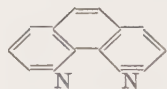
THE EFFECTIVENESS OF CHEMICAL AGENTS which presumably modify radiation toxicity in whole or in part by direct interaction with the copper of the critical copper oxidases should be a function of their chelating ability as measured by the stability constant. Consequently, changes in molecular structure which decrease or eliminate the ability to chelate with Cu(I) should result in a loss or decrease in radiobiological activity of the chemical agent. Many compounds may be metabolically altered and if so, chelating groups may be removed, modified, or replaced. On the other hand, a compound incapable of chelation may have ligands incorporated which impart chelating properties to the compound. In any event the Cu(I, II)-Peroxy theory utilizes the chemical information available on the stability of the cuprous and cupric chelates for a given compound to predict its radioprotecting or radiosensitizing potential. In order for a chemical agent to form a complex or chelate with enzyme-bound copper the $\log K_1$ must exceed 6 as a minimum assuming that the chelating reaction involves the displacement of hydroxide or chloride ion (see Table II.2).

Various investigators have suggested that copper is involved in radiation damage (Chapter V). Of interest here is the work of Knoblock and Purdy (K17) who measured the stabilities of complexes formed between copper and several compounds including the mercaptoamines. However, they did not differentiate between the cuprous and cupric preferences of the compounds tested. Fallab and Erlenmeyer (F1a) in a discussion of chelation and copper as catalysts in radiobiology commented on the fact that in a homologous series of protective agents, practically no

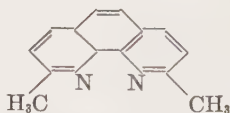
radioprotection was observed with 4-mercaptobutylamine—a point discussed in the following section of this chapter.

The fact that a compound is incapable of reacting with copper means that if it does modify radiation the mechanism does not involve copper interaction. But the fact that a compound can react strongly with copper does not automatically mean that it will modify radiation toxicity. It may, for example, be unable to penetrate to the critical sites. Presumptive evidence of direct Cu(I) interaction exists when a protective compound with marked copper (I) chelating ability loses its modifying action when the copper (I) reactivity is eliminated or markedly reduced following structural changes. Experimental criteria for confirming the mechanism should then include observations such as the measurement of *in vivo* inactivation of cytochrome oxidase of radiosensitive tissues and ability to protect against neutron irradiation (p. 178).

The geometry of the copper site in a protein or enzyme and the structure and size of a complexing agent determine whether contact between the copper and the compound will be made. While many ligands react very strongly with copper, only the small cyanide ion is known to inhibit all the copper enzymes. The presence of simple but bulky methyl groups in a compound can make a big difference as, for example, in the following two compounds:



1, 10—Phenanthroline



Neocuproine

Ceruloplasmin is inhibited by 1,10-phenanthroline but not by Neocuproine (F11). Similarly, the incorporation of two methyl groups in cysteine to form penicillamine probably prevents the metabolically more stable penicillamine from producing *in vivo* inhibition of liver cytochrome oxidase and accounts for its lack of radioprotective action (see Chapter XI).

Many other examples of relationships between the size and shape of a chelating agent and the degree of reactivity with cop-

per enzymes and proteins are known, especially for cytochrome oxidase (p. 48). It may be that the copper atoms are buried below the surface of the protein—a result paralleled by the “crevice theory” of St. George and Pauling (M4, p. 198). Naturally, different copper enzymes react differently toward the same reagents. Thus, EDTA inhibits ascorbic acid oxidase rapidly but requires several hours to inhibit tyrosinase and has no effect on cytochrome oxidase (F11, p. 482).

As discussed elsewhere (p. 87), there are many uncertainties involved in the assay of cytochrome oxidase even if no problem of contact between copper and the chelating agent existed. Therefore, there exist severe limitations in using the ability of a compound to inhibit cytochrome oxidase *in vivo* as a means of screening potential radioprotective agents. For example, during the homogenization of a tissue in preparation for assay of enzyme activity, the release of cell constituents and enzymes may destroy a compound such as cysteine, thus destroying the bond presumably formed between cysteine and copper and reactivating the enzyme.

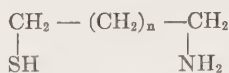
Several studies have been made on the distribution of radioactive labeled chemical protective agents of the cysteine-cysteamine group (E6, H12, M1). In all cases it was found that these compounds penetrated the intracellular compartments including the cytoplasm and nucleus.

One of the best examples of the need to consider intracellular distribution and penetrability involves the D and L isomers of mercaptobutyl-2-guanidine. These optical isomers differed considerably in protective action although their tissue distribution was identical (S44a). However, the degree of protection with these and some other protective drugs appeared to be correlated consistently with the intracellular distribution in microsomes of liver of the mouse, rat and spleen of the rat.

Chain Length and Branching

Such well-known protective agents (administered prior to irradiation) as the mercaptoalkylamines and the aminoalkylisothiuronium compounds have been extensively tested by Doherty and co-workers (M1, D15, S30) using mice receiving a lethal

dose of x-irradiation. The mercaptoamines having the general formula:

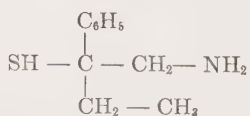


Mercaptoalkylamine

where $n = 0$ and $n = 1$ show high protective activity, while the derivative with $n = 2$, namely 4-mercaptobutylamine is only slightly active, a fact confirmed by Tank (T2). Similarly the S,4-aminobutylisothiuronium compounds are found to possess only slight protective activity as compared to the corresponding propyl and ethyl derivatives. These results accord with the Cu(I, II)-Peroxy theory. When $n = 2$ a relatively weak 7-membered chelate ring is formed with the cuprous ion. The estimated $\log K_1$ for such a 7-membered ring is roughly 6—a drastic decrease from a stability constant of about 18 log units for the corresponding 5- and 6-membered rings.

At least two other facts must be considered other than the stability constant when the chain length or type is modified. All things being equal, a compound which can penetrate the intracellular sites where the copper enzymes are located would be expected to possess a greater protective activity. On the other hand, the chemical toxicity increases for related reasons. Thus, 2-mercaptopropylamine (MPA) which forms a 6-membered chelate ring with Cu(I) of high stability is found to be more effective than 2-mercaptoethylamine (MEA) on the molar basis but the toxicity of MPA is relatively greater than MEA so that the net gain is small (S30).

Because of steric interference (M8) the ability of a mercaptoalkylamine to react strongly with Cu(I) may be reduced even if the chain length is suitable. A good example is provided by the compound:



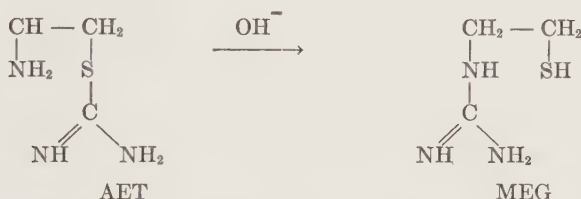
2-mercaptoalkyl-2-phenyl-2-ethylamine

studied by Tank (T2). The presence of the fully substituted

β -carbon atom next to the sulfur atom hinders the formation of a cuprous chelate and ability to react with cytochrome oxidase and results in a marked decrease in stability. On the other hand, the replacement of only one hydrogen of the β -carbon atom by an alkyl group as in 2-mercapto-2-methyl ethyl amine could be beneficial since the penetrability of the compound would be expected to increase without a concomitant decrease in the stability of the chelate.

Structures Requiring Rearrangement

The compound, 2-aminoethylisothiurea (AET) rapidly and quantitatively transforms into 2-mercaptoethylguanidine (MEG) upon neutralization (S30).



Experimentally it is found that MET provides effective protection to mice given lethal doses of x-irradiation.

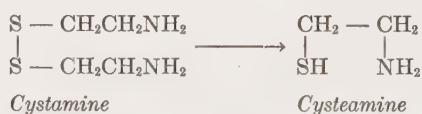
On a chemical basis, AET is incapable of forming a chelate with Cu(I) but the rearrangement product forms a strong chelate ($\log K_1 \approx 19$) with Cu(I) but not with Cu(II).

A simple chemical test to demonstrate the lack of interaction of AET with copper is easily made. To a dilute solution of cupric chloride is added the acid salt of AET. No interaction is noted. However, as the solution is made alkaline with NaOH the cupric ion is chelated as shown by the formation of the yellow cuprous color. The resulting chelate is stable in alkaline pH. The necessity for the presence of a free sulfhydryl group for the reduction of cupric to cuprous concomitant with the formation of the chelate is shown by the observation that the addition of the acid salt of penicillamine, β, β -dimethyl cysteine, to cupric chloride does result in an immediate color change from blue to yellow. The strength of the cuprous-MEG chelate is shown by the fact that the return of the blue cupric ion can only be effected with a cupric chelating agent possessing nearly

equivalent chelating strength such as EDTA ($\log K_1$ for cupric-EDTA 18.8). Similarly, the addition of MEG or penicillamine to the intensely blue EDTA chelate is capable of discharging the blue color in favor of the yellow cuprous color. These color changes can be repeated, showing that they are chemically reversible.

Compounds such as cystine and oxidized glutathione do not form chelates with Cu(I) because of the disulfide linkage. However, oxidized glutathione forms a stable 1:1 chelate with Cu(II) through the amino and carboxylic groups. Hence, neither cystine or oxidized glutathione can possess any protective action involving direct Cu(I) interaction unless the disulfide ring opens so as to make an -SH group available.

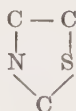
Unlike cystine, the corresponding disulfide, cystamine gives good protection to irradiated mice. Insofar as copper interaction is involved, cystamine cannot be active unless it reduces to the corresponding sulfhydryl compound, cysteamine which would be capable of reacting with the cuprous ion according to the reaction:



Experimentally (B6), it has been demonstrated that cystamine is reduced to cysteamine both *in vivo* and *in vitro*.

Similarly, those isothiuronium derivatives which cannot rearrange to the free thiol would also be practically devoid of protective ability. The cyclic compound, o-aminothiazoline, has protective activity only because the ring opens (S30).

Another group of compounds, the thiazolidines having the skeleton have been shown to provide protection to mice (K1).



The fact that some thiazolidines readily hydrolyze to mercaptamines and are then able to react with copper presumably ex-

plains their protective action. Further discussion of these compounds is given in Chapter XII.

Substitutions on Donor Ligand Atoms

Consider the effect of substituents on the amino nitrogen atom in the mercaptoamines and isothiuronium compounds from the standpoint of chelation. In general, it has been found that the more available the electron pair of the nitrogen the stronger is the covalent bond formed with copper. Hence, electron releasing groups would make the nitrogen more negative and increase the availability of the donor electrons while electron-attracting groups would have the opposite effect. In other words, substituents which make the nitrogen more basic would tend to increase the stability of the chelate while those tending to reduce the basicity of the nitrogen would weaken the stability.

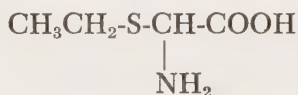
Chaberek and Martell (C4) have pointed out that both hydrogen ions and metal ions are Lewis acids, that is, electron acceptors, and that the interaction of a metal ion with a base corresponds to a neutralization reaction in exactly the same sense as does the corresponding reaction involving hydrogen ions (see reference (K4) also). Therefore, insofar as chelating strength is concerned, substitution of an acetyl group on the amino nitrogen in the mercaptans or the isothiuronium compounds should cause them to lose all or part of their protective action insofar as it is due to combination with the target copper atoms. This is in agreement with the experimental facts as has been demonstrated in the detailed experiments of Doherty *et al.* (S30) with the N-acetyl derivatives of AET involving mice in which he showed that the acylaminomercaptans have greatly reduced protective activity or none at all.

The electron-repelling alkyl groupings will tend to enhance the basicity of the amino nitrogen and such derivatives would be expected to retain protective activity. While this appears to be the case, the chemical toxicity is sometimes too high to permit the administration of amounts equivalent on a molar basis to the unsubstituted mercaptoamines. On the other hand, substitution in the strong electron-repelling guanido group appears to en-

hance the protective activity in the particular test system involving mice employed by Doherty (S30).

Increasing substitution on the amino nitrogen diminishes the tendency for coordination even if the substituent is electron-repelling (M8). Consequently, it would be expected that trialkylamino compounds, for example, would be ineffective as, indeed, they are (S30).

Substitution on the sulfur atom to form a thioether gives agents which react only very weakly with copper ($\log K_1 \sim 4$). Thus it is expected that thioethers will be largely ineffective as protective agents unless of course the thioether was converted to a sulfhydryl group by metabolic processes—as in AET discussed earlier. The experimental data demonstrate that thioethers are usually ineffective. The compound, S-ethyl cysteine



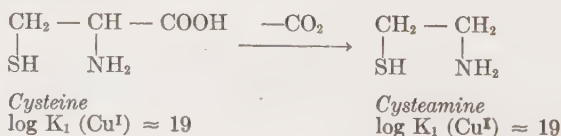
S-ethyl cysteine

for example, is completely ineffective as well as methionine and other compounds of this nature (D18, P9).

Changes in the Type or Number of Functional Groups

The effect of changes in the type and number of functional groupings on the stability constant of copper chelates can be evaluated or estimated. It is possible to change or switch functional groups without affecting the stability of the copper chelate. However, the resulting compound may switch from a preference for Cu(I) to a preference for Cu(II) which could result in a loss of protective action and the acquisition of a sensitizing action.

Certain changes in a molecule have relatively little effect on the stability constant. For example, decarboxylation of cysteine to form cysteamine:



aside from possible metabolic consequences, has little effect on the stability of the cuprous chelate formed with the -SH and -NH₂ groupings because the corresponding cupric chelate involving the -NH₂ and -COOH groupings is far weaker ($\log K_1 (\text{Cu}^{\text{II}}) \approx 8$). Therefore, it would be expected that the effect of decarboxylation might improve the protective action mainly because the carboxyl group would tend to enhance the water solubility of the compound and thus reduce the possibility of intracellular penetration. On this basis, esterification of the carboxyl group in cysteine to form, for example, the cysteine methyl ester, might be expected to enhance the activity relative to the parent compound. Experimentally, cysteamine and the ethyl ester of cysteine possess excellent protective action and according to Doherty (D15) they are superior to cysteine *for the organism tested*. However, insofar as the therapeutic index is concerned this is not necessarily true. It must be always kept in mind that the very factors which lead to enhanced penetrability usually enhance toxicity.

Another change which in general would be expected to reduce the stability of the copper chelate and hence the radiobiological action would be the substitution of a hydroxyl group for the sulfhydryl group. Replacement of the -SH group with an -OH group will drastically reduce the copper binding ability and also abolishes any radiobiological activity involving direct copper interaction (A4) as, for example, ethanolamine.

These observations are summarized below:

$\begin{array}{c} \text{CH}_2 - \text{CH}_2 \\ \quad \\ \text{SH} \quad \text{NH}_2 \end{array}$	$\begin{array}{c} \text{CH}_2 - \text{CH}_2 \\ \quad \\ \text{OH} \quad \text{NH}_2 \end{array}$
<i>2-Mercaptoethylamine</i>	<i>Ethanolamine</i>
$\log K_1 (\text{Cu}^{\text{I}}) \approx 19$	$\log K_1 (\text{Cu}^{\text{I}}) \approx 3$
PROTECTIVE	INEFFECTIVE

Replacement of the amino group in 2-mercaptoethylamine with a hydroxyl group can be expected to produce some lowering of the stability of the copper complex since the sulfur-copper-oxygen bond is weaker than the sulfur-copper-nitrogen bond (M18, p. 16). In any event, for the concentration ranges tested, mercaptoethanol has little protective action (D18, K17). In this

connection it is interesting to note that 1-thioglycerol is protective but not mercaptoacetic, mercaptosuccinic acid, or dithiodiglycerol (D18).

However, it is difficult to interpret the data on compounds involving sulfur-copper-oxygen bonds unless they are tested over a wide range of doses. What may be more important is the fact that little is known of their metabolism in a given species. For example, it is known that in the rat the compound, β -mercaptopyruvic acid is broken down very rapidly releasing sulfur from the sulfhydryl group (W10, p. 395).

SULFHYDRYL COMPOUNDS AND ANTIOXIDANT ACTION

Many structural changes have been made in sulfhydryl compounds which eliminate much of their chelating ability. Yet the fact that they lose their protective action but still retain antioxidant properties is *a priori* evidence for direct Cu(I) interaction. It is probable, nevertheless that part of the radioprotective action of the readily reducible sulfhydryls involves a lowering of oxygen levels. However, the antioxidant action of sulfhydryl compounds in mammals is small and can only be effective in local areas. Thomson (T7), for example, points out that the protective dose of cysteamine in mouse would consume 280 microliters of oxygen when converted to cystamine or 1680 microliters if completely oxidized to taurine. The oxygen consumption of the resting mouse is about 600 microliters a minute.

Chapter IX

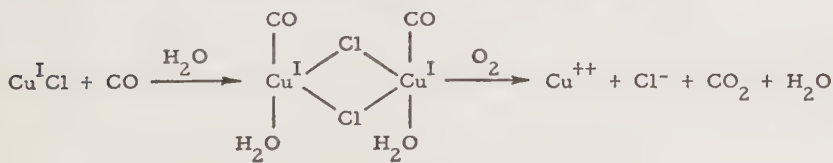
PROTECTION BY CARBON MONOXIDE, NITRIC OXIDE, REDUCING AGENTS, AND AMINES

COMPOUNDS such as the monothiols, monocarboxylic acids and monophenols react only weakly with copper as compared to their polyfunctional counterparts. According to the Cu(I,II)-Peroxy theory, if these monofunctional compounds do modify radiation toxicity it can *only* be due to pharmacological action in which, for example, oxygen deficiency is induced. The experimental data available in the literature bear out these expectations. The list of compounds tested is a long one (D18, E6, L1, T7) and includes the thiophenols, thioglycollic acid, 2-mercaptopropionic acid, thiolacetic acid, pyruvic acid, diphenylamine, ethanethiol, and phenols.

In this chapter are described the actions of some of the more widely used and interesting simple chemical protective agents. Their effectiveness is presumed to be due to direct copper interaction and in addition, or solely, to interaction with peroxides and free radicals.

CARBON MONOXIDE

Carbon monoxide interacts preferentially with cuprous copper but the resulting complex readily undergoes disproportionation with the net result that the oxidation of the cuprous copper to cupric copper takes place. The reaction of carbon monoxide with cuprous copper is depicted (S33) as follows:



The ready dissociation of the carbon monoxide complex by oxygen or ionizing radiation helps clarify the radiobiological actions of carbon monoxide.

In mammals carbon monoxide would be expected to produce an oxygen deficiency within the tissues because its principal metabolic fate is to combine with hemoglobin to form carboxyhemoglobin. Carbon monoxide has been found to possess radioprotective action in animals when about 60 per cent of the hemoglobin has been converted to carboxyhemoglobin, thus hindering the transport of oxygen (B6). This fact does not necessarily prove that the gross interference with oxygen transport is the sole mechanism by which CO protects irradiated animals. For example, both p-aminopropiophenone (PAPP) and sodium nitrite produce methemoglobinemia in rats and mice but only PAPP appears to protect (D20). In actuality, a small but definite fraction of CO reacts with intracellular components, especially cytochrome oxidase. However, this reaction is probably of minor consequence relative to the interference with oxygen transport insofar as radioprotection is concerned because the presence of oxygen or ionizing radiation could lead to rapid dissociation of the cuprous-carbon monoxide complex. In fact, while CO inhibits cytochrome oxidase, the effects are readily reversible by oxygen (H1, K7). Nevertheless, the inhibition of cytochrome oxidase may be responsible for the small degree of protection afforded by CO when administered after irradiation (K21a, also see Chapter XII).

It would appear that part of the chemical lethal action of carbon monoxide which involves inhibition of copper oxidases would be readily reversible by administration of oxygen or ionizing radiation. This leads to the curious expectation that ionizing radiation could be an antidote to carbon monoxide poisoning. In fact, Cameron (C1) has demonstrated that x-rays delivered to rats and monkeys enable them to survive an otherwise lethal dose of carbon monoxide! The subsequent radiation injury is disregarded but, of course, eliminates x-rays as a practical therapy for CO poisoning.

NITRIC OXIDE

In *dry* systems nitric oxide protects dry spores and seeds against damage by ionizing radiation (D13, P13-15). The sensitizing action of nitric oxide in *wet* systems is described in Chapter XI. As a scavenger for oxygen and free radicals the NO protects the radiosensitive receptor sites including the copper containing enzymes from attack. In the absence of water, and unlike oxygen, the formation of nonradicals by NO is facilitated, as for example:



It should be pointed out that many of the rate and thermal responses involving NO in other gases in dry systems are probably determined nearly entirely by diffusional processes. In fact, the activation energies observed in these systems closely parallel those observed for the diffusion of molecules in polymer systems (B14). It would be interesting to apply the type of reasoning and calculations described by Barrer (B14) to these dry systems in which the species diffusing can be considered, for example, to be organic free radicals and interacting molecules.

REDUCING AGENTS AND POLYPHENOLS

According to Bacq and Alexander (B6) the protective action of many drugs is supposed to be linked to their reducing action. They point out, however, that ascorbic acid is practically ineffective while cysteine, diethyldithiocarbamate and other reducing agents are good protectors.

The actions of reducing agents can be evaluated in terms of copper interaction. Thus, for *in vivo* protection a reducing agent must be capable of: 1) reducing Cu(II) to Cu(I), 2) forming a firm complex or chelate with the Cu(I) receptor, and 3) of contacting the enzymatic copper. Neither ascorbic acid, or other reducing agents such as sulfite, or sodium borohydride can be expected to be effective *in vivo* because they have no appreciable ability to combine with Cu(I); they tend to remain extracellular so that it is difficult to sustain or obtain suitably high concentrations in the vicinity of the receptor; and are rapidly autooxidiz-

able. In *in vitro* suspensions (e.g., bacterial) where the oxygen supply is limited and high concentrations of the reducing agent can be maintained, protection by non-chelating reducing agents is often observed. The possible role of the hydrated electron in relation to the sensitizing action of some carbonyl compounds is discussed elsewhere (p. 171).

All of the reducing agents which do possess good protecting ability in mammals do form firm chelates or complexes with Cu(I). In these cases, variation in effectiveness is a matter of relative excretion rates, toxicity, and metabolic and chemical stability.

It is difficult to predict the action of polyphenols on radiation toxicity unless knowledge as to the specific chemical reactions proceeding at the enzyme site is available. However, the possibilities can be given in specific chemical terms. Thus, it is unlikely that monophenols (aside from their antioxidant action, i.e., peroxy destruction) would be effective inasmuch as they do not undergo marked interaction with copper, either through complex formation or as reducing agents. However, the polyphenols in which the phenolic groups are ortho to each other are capable of undergoing catalytic oxidation by copper enzymes (M8, M12). During the catalytic action, Cu(II) first forms a firm chelate with the polyphenol following which the Cu(II) is reduced temporarily to Cu(I). If the concentration of a polyphenol or polyhydroxyl compound could be maintained in high concentrations at the copper site in copper oxidases, and if the particular compound could serve as a substrate for the enzyme, then some protection might be found. The protective action would be weak, however, because of the very weak binding to Cu(I) and the hydrophilic properties of the -OH groups. Lacassagne and co-workers (L1) have tested numerous phenolic compounds for protective action against whole body irradiation in mice and some protection appeared to be observed only when a long fatty acid chain was attached to the molecule ($C_6 - C_{22}$). The fatty acid chain would serve to enhance the intracellular concentration of the phenol.

AMINES

Aside from their possible and at times demonstrable pharmacological effects (D20) it would be expected that amines

could modify radiation injury by direct chemical interaction with radiation produced peroxides (p. 69). In general, however, the reactions of amines with peroxides can be expected to exert a limited radioprotective effect because of metabolic limitations. However, they offer interesting possibilities for protection against neutron irradiation and for chemical post-irradiation therapy (p. 112).

Alexander, Bacq and associates (A4, B6) have tested the protective effects of many amines on the survival of mice given lethal doses of x-rays. The results were variable but they found that compared to no survival in the control mice, the percentage survival in treated mice were as follows: methylamine (30 to 70%); ethylamine (10%); allylamine (0%); dimethylamine (0%); ethanolamine (0%); triethanolamine (0 to 20%); β -phenylethylamine (80%); and aniline (10 to 40%).

Little or no protective action is afforded by trimethylamine, trimethylamine oxide, or by tetra-ethyl ethylene diamine (S24). In most cases where negative results are obtained it appears that failure is due to the metabolic destruction or alteration of the amine, preventing its reaction with the radiation produced peroxides. In the case of trimethylamine, for example, it is known that it is converted *in vivo* rather efficiently to trimethylamine oxide by enzymatic action of trimethylamine oxidase (W10, p. 142). Other reactions include the degradation of trimethylamine to ammonia and subsequently to urea.

In principle, if a metabolic stable amine is administered which can also penetrate intracellularly, it should possess the ability to modify radiation toxicity by destruction of peroxides.

From a chemical and metabolic standpoint, it would appear that one of the most successful radioprotectants would be ethylenediaminetetraacetic acid:



and related compounds. These are tertiary amines.

Bacq *et al.* (B5) have demonstrated that EDTA protects mice from lethal doses of irradiation but as was pointed out elsewhere (Chapters V and XII), the protection was obviously due to other causes associated with the administration of near lethal, hypocalcemic doses of the sodium salt of EDTA rather than to a chela-

tion mechanism. In unpublished experiments (S24) it has been found that giving an equivalent molar dose of the calcium-sodium salt of EDTA provides a small though definite degree of protection when administered prior to irradiation. The results are rather erratic, however, varying from zero to about 40 per cent protection. Combining the results of several experiments in which doses of EDTA varying from 150 to 1,000 mg/kg were employed, it was found that out of 40 controls only 2 mice survived (5%) a standard dose of 750r compared to a survival of 12 out of 80 treated (15%). With a near toxic dose of 2,000 mg/kg of the calcium-sodium salt of EDTA, 50 per cent of the treated mice survived compared to 10 per cent in the controls.

It appears that EDTA can protect by destroying peroxides through a chemical reaction typical of tertiary amines. In this connection it is interesting to note that EDTA destroys peroxides in solutions at pH 7 (F11). It has also been found to inhibit lipid peroxide formation in rat liver homogenates and in rat liver mitochondria exposed to UV (B11). In these cases it would appear that EDTA might act by inactivating a metal catalyst.

Since EDTA is highly water soluble, it is suggested that esterification of the molecule to enable it to penetrate intracellular regions would, combined with its high metabolic stability, provide more efficient protection against radiation injury. A discussion of the radiobiological action of EDTA when combined with a nitrile for post-irradiation therapy is discussed in Chapter XII.

Chapter X

PROTECTION BY MODIFICATION IN ENVIRONMENT OR PHYSIOLOGICAL AND METABOLIC STATE

IT IS WELL KNOWN that radiobiological responses of living organisms are modified by changes not involving chemical treatment such as diet, oxygen tension, water levels, metabolic state, and age. The purpose of this chapter is to examine the extent to which some of these radiological responses may be explained by the Cu(I,II)-Peroxy mechanism.

HIBERNATION

Hibernation occurs in a small number of mammalian species and consists of a profound drop in body temperature with a concurrent decrease in metabolism and heart rate (L19). The effects of ionizing radiation in hibernation have been reviewed by Smith (S38). Experiments on the response of several species to x-irradiation while hibernating and kept in hibernation for varying periods of time thereafter have shown that the expression of radiation damage is markedly slowed during hibernation. If after the hibernation periods the animals are removed to and kept in a warm environment, death occurs as in the case of non-hibernating animals irradiated and kept in the warm environment (S38).

In general, cellular damage proceeds even during hibernation following irradiation but species differences on the rate and extent of this damage occur. Protection against the lethal effects of irradiation during hibernation is obtained with certain sulfhydryl compounds when these are administered before but not after irradiation. However, in one case, that of the dormouse, protection by cysteine has been reported when the animals were

treated while hibernating or even three weeks after irradiation (K19).

According to Smith (S38) it seems possible

... that the low temperature of the hibernating dormouse may, because of different radiosensitivity of enzymes and different activation energies of enzymes leading to alternative metabolic pathways, bring about biochemical changes unlike those encountered in the homeothermic animal in that they can repair by cysteine. In the absence of administration of cysteine these biochemical changes are ultimately expressed as death.

A more specific explanation of the protective effects of hibernation can be given based on two experimental facts: (1) The decrease in organic peroxide levels with time (p. 93); and (2) The fact that the copper oxidases are resistant to radiation damage or peroxide attack when in the oxygenated state (p. 84).

In the case of the copper oxidases, and specifically cytochrome oxidase, the steady state values, namely the ratio of the oxygenated to de-oxygenated forms, is very large—greater than ten to one (C5). If the steady state values are increased it means that the proportion of the time the enzyme is in the radiation resistant or oxygenated state is greater. Consequently the damage to the enzyme system by radiation would be less under conditions in which the steady state values are higher.

In the hibernating animal the oxygen concentrations in the tissues remain high but the utilization of oxygen is far less. The metabolic rate of animals in hibernation is about 1/30 to 1/100 of the "resting" metabolic rate when the animal is in the homeothermic state (L19). During the period of arousal there is a great burst of oxygen consumption. These factors suggest that one of the factors for the protective effect of hibernation is due to the slower turnover of oxygen. Thus, the enzymes exist in a radio-resistant form for a much longer period than in the non-hibernating animal. A diagram illustrating this situation is depicted in Figure X.1.

A concomitant factor contributing to the protection afforded by hibernation is the disappearance of the organic peroxides. Consequently, if the hibernation period is long enough following

HIBERNATION AND RADIOBIOLOGY.

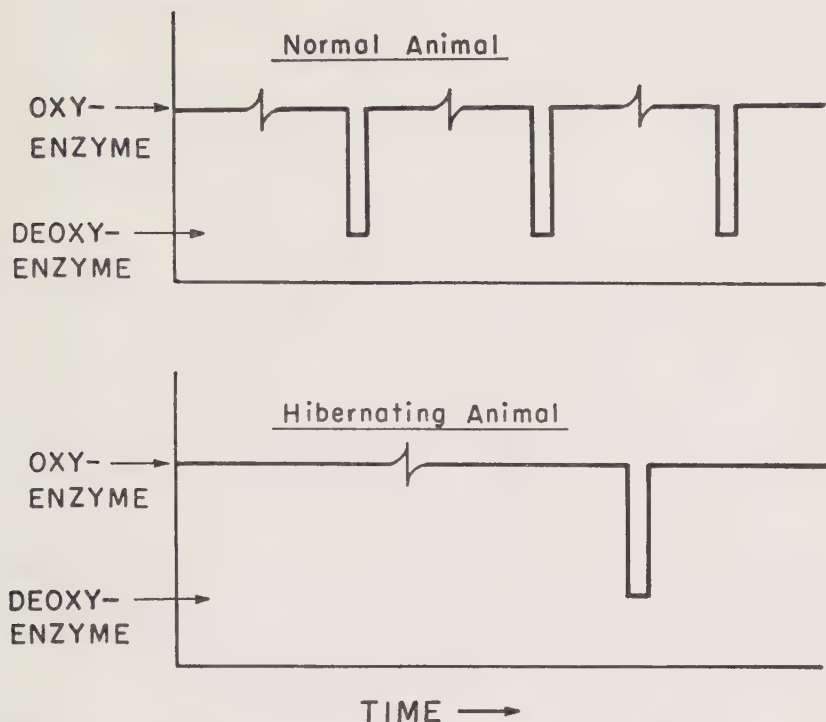


Figure X.1. Schematic diagram illustrating the postulated differences in the ratio of the oxygenated forms of the copper oxidases in normal animals compared with hibernating animals.

irradiation, the subsequent damage from radiation is expected to be diminished. However, the excessive peroxides produced by irradiation in the ordinary animal at room temperature remains elevated for several days (H14, M3) as discussed in Chapter IV. At the lower hibernating temperatures it would be expected the lifetime of the radiation produced peroxides would be far longer. In the case of irradiated squirrels maintained indefinitely in the poikilothermic state, mortality eventually occurs (E7a, p. 302). This may mean that at a certain point irreversible damage has taken place regardless of the ultimate level of peroxides.

Many possibilities exist for chemical protection of irradiated hibernating animals. These include the use of scavenging agents

for peroxides such as the cyanides and nitriles to protect the enzyme during the period it becomes de-oxygenated. The protective effect of cysteine when given post-irradiation to the hibernating dormouse may reflect a markedly reduced destruction of cysteine by enzymatic action, e.g., amino acid oxidase (A11). Consequently, when the animal is awakened from hibernation it is conceivable that sufficient cysteine remains to act as a protective agent.

Insofar as peroxy radical formation and destruction is concerned, certain physiological consequences of hibernation which may have a bearing on radiation toxicity include the fact that during hibernation the body fat becomes desaturated. A non-hibernating animal such as the albino rat does not desaturate its fat when exposed to low environmental temperatures (L19). The remarkably low heart rate of the hibernating animal may inhibit the transport of organic peroxides via the circulation. The respiratory quotient of the hibernating animal is very close to 0.7, indicating that it is using fat almost exclusively during hibernation. This destruction of the fat stores may also lead to diminished peroxide production with time by consuming the substrate necessary for the autooxidation process to occur.

AGE AND RADIOSENSITIVITY

Intact Animals

The radiosensitivity of new born mammals and old mammals is higher than those of adult mammals (B7, p. 299). During full adulthood the radiosensitivity remains fairly constant. In the rat the LD₅₀ at age 3 months is roughly double that at 3 weeks. After 3 months the LD₅₀ diminishes with age (R4, p. 123). It might be anticipated that cytochrome oxidase levels would parallel the variation of radiosensitivity with age as has been observed for some mammalian tissues but not including the kidney and brain (K24).

A careful study of the variation of radiosensitivity of mice with age has been made by Crosfill, Lindop, and Rotblat (C15). Their data have been plotted along with the variation with age of the cytochrome oxidase activity of rat and mice livers. Crosfil

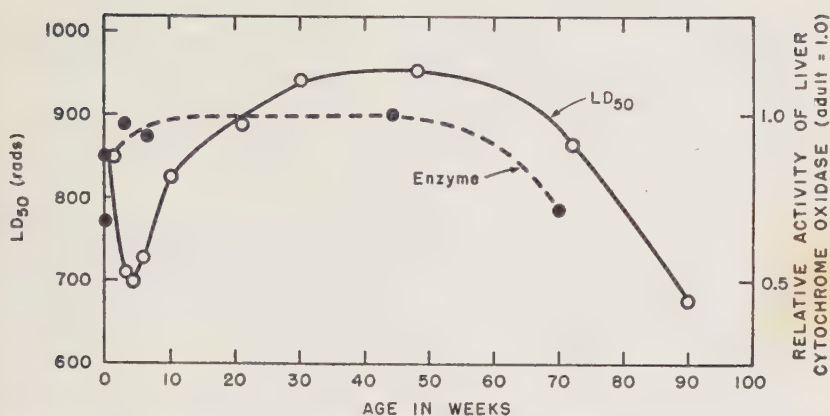


Figure X.2. Variation in the radiosensitivity of mice with age (from C15) and the corresponding variation in the levels of cytochrome oxidase activity in the livers of mice and rats.

et al. (C15) remark that their data on radiosensitivity with age shown in Figure X.2 is particularly noticeable at young ages. They go on to say that none of the theories which have been put forward to explain the effects of radiation account for this variation. It is interesting to note, therefore, that the cytochrome oxidase levels also show remarkable changes at very early ages. Available data on cytochrome oxidase levels, however, are fragmentary, so it was necessary to combine data from several sources (D19, D5, P12, S4). The parallelism between radiosensitivity, age, and cytochrome oxidase levels in the liver is suggestive (Fig. X.2 and Fig. IV.2) and indicates that it would be worthwhile to obtain systematic measurements of the variation in cytochrome oxidase levels with aging for different tissues as a basis for further explorations of this problem. The liver is probably the poorest tissue to test in this connection.

The newborn rat is highly resistant to anoxia in comparison to the adult, and in the course of 10 to 20 days loses this resistance and becomes as susceptible as the adult. From these and other data on oxygen uptake, Potter *et al.* (P12) comments:

. . . The resistance of the newborn rat to anoxia suggests a relative independence from aerobic processes in comparison with adult specialized tissue. In this respect it is similar not

only to embryonic tissue . . . but to cancer tissue as well. The fact that the newborn rat brain is embryonic in type, and that the conversion to the adult type of metabolism is compressed into the short space of about 15 days, facilitates the demonstration of the enzymatic changes that occur during this period of development.

It would be interesting to determine the relative radiosensitivity of the guinea pig with age inasmuch as this species is nearly as sensitive to anoxia at birth as the adult. In fact, the resistance to anoxia appears to vary inversely with the degree of maturity at birth.

The above observations suggest that the variation of radiosensitivity with age in young animals would be a function of the resistance to anoxia and more specifically, to cytochrome oxidase activity once the anaerobic capability becomes minor. Consequently, it would be worthwhile to check these points by suitable experiments with different species *in utero* and after birth for a period long enough for the cytochrome oxidase activity to have become relatively independent of age.

In view of the close relationships between vitamin E levels and *in vivo* peroxide production (p. 95) it is apparent that the variation of vitamin E levels, and hence peroxide production with age can be a significant factor in radiosensitivity. It is known that tocopherols, which are an index of vitamin E levels, are widely distributed in human tissues from early fetal life to advanced old age, and that their concentration varies with age, being low at the very young and very old ages (M13).

Embryonic and Neoplastic Tissues

In general, very young and rapidly growing cells contain fewer mitochondria than adult or older cells. Concomitant with the decrease in mitochondrial number goes a decrease in cytochrome oxidase activity (G9). It is suggestive to note that these changes in mitochondrial number and of cytochrome oxidase activity generally parallel changes in the radiosensitivity. However, in the absence of parallel and control experiments on peroxide levels as a minimum, it would be naive to expect that relative cytochrome oxidase levels alone necessarily reflect relative radio-

sensitivities. Be that as it may, it is interesting to review some of the known information on mitochondrial and cytochrome oxidase changes in tissues with age.

Dawkins (D5) has pointed out that a great deal of biochemical data suggest that individual liver cells in the fetus contain fewer mitochondria than in the adult. During the induction of liver tumors with butter-yellow or thioacetamide, a fall in the mitochondrial population is found (G4). Many, but not all tumors have been found to have a low population of mitochondria per cell, and, as Dawkins points out, this may be a common characteristic of rapidly growing tissues.

During the maturation of the rat brain the increase in "mitochondrial capacity" is entirely due to an increase in number of mitochondria rather than in size or composition (S2). In terms of the number of mitochondria per cell, the one-day-old rat brain has 286 mitochondria per cell by one method of measurement, and this number increases to 506 by the 10th day, to 1036 by the 21st day and to 1328 by the 50th day. All in all, 60 per cent of the protein increase in the brain is from the rise in the number of mitochondria. These observations are suggestive and are well worth experimental investigations to determine the extent to which the variation in radiosensitivity of the brain with age is governed by these increases in mitochondrial number. A discussion of mitochondrial number with respect to tissue sensitivity is given on page 186.

INERT GASES AND OXYGEN-DEPENDENT RADIOSENSITIVITY

It has been shown that the inert gases, He, A, Kr, and Xe, when present during irradiation in sufficient concentration can counteract the action of O_2 in at least two materials—the main root of the broad bean, *Vicia faba*, and mouse Ehrlich ascites cells *in vitro* (E1, E2). The criterion of damage in the bean root was the reduction in growth rate after irradiation, and in the ascites cells the reduction in the number of normal cells scored in the first division after irradiation *in vitro* and reinoculation into untreated host mice. Other gases, H_2 , N_2 , N_2O , and C_3H_6 show similar activity. There is no qualitative difference in the damage

caused by irradiation in the presence or absence of oxygen, the oxygenated state simply acting as an enhancement factor.

The evidence given by Ebert *et al.* (E1) suggests very strongly that the inert gases act intracellularly. They postulate:

. . . that specific and finite sites exist within the cell at which oxygen must be present to confer oxygen-dependent sensitivity, and that inert gases can displace oxygen from these sites. That oxygen and the other gases compete for the same sites is indicated by the experimental finding with bean roots that sensitivity is governed by the ratio of oxygen to the other gas provided that the partial pressure of oxygen is sufficient to guarantee saturation of the site. Furthermore, when the oxygen effect was blocked by one gas . . . the addition of an excess of another protecting gas . . . did not further reduce the radiosensitivity. This result suggests one mechanism for both gases, and is consistent with a displacement adsorption phenomenon at a defined site . . .

It is well-known that inert gases possess anesthetic action and it had been postulated that this action was related to their lipid-water partition coefficient (E1, S41). Accordingly, it was ascertained (E6) that a similar correlation relating lipid-water solubility and radiosensitivity existed (Table X.1). It should be kept in mind that not only is the lipid-water ratio important but also the absolute solubility—which explains the apparent discrepancy involving nitrogen and argon shown in Table X.1.

Ebert (E1) suggests that the site from which oxygen is displaced may be associated with some lipid-like material in the cell. Aside from the fact that part of the effect may be within the nucleus, it is postulated here that the critical site resides within the mitochondria. Since the lipid component of the mitochondrial membrane consists of a bimolecular lipid layer (Fig. IV.1) inert gases are presumed to displace oxygen from the lipid membrane by virtue of their high solubility in this medium. Consequently the radiation yield of peroxides within the mitochondria is decreased.

LIPIDS AND ANTIOXIDANTS

A considerable amount of literature has arisen on the effects of fats on irradiated organisms (A13). It has been shown (C6,

TABLE X.1

EFFECTIVENESS OF VARIOUS GASES IN REDUCING THE OXYGEN-DEPENDENT
RADIOSENSITIVITY OF *Vicia faba* ROOTS^a

Gas	Molecular Weight	Bunsen's Absorption Coefficient ^b		Oil/Water Solubility Ratio	Pressure in atm. for 50% Effect ^c
		Water	Olive Oil		
Helium	4	0.0085	0.015	1.7	55
Neon	20.2	.0097	—	—	—
Nitrogen	28	.013	.067	5.2	12.5
Argon	39.9	.026	.14	5.3	2
Krypton	83.7	.045	.43	9.6	2
Xenon	131	.085	1.7	20.0	1.1
Radon	222	.15	19.0	125.0	—

^a Adapted from data on radiosensitivity of Ebert *et al* (E1) and of Tobias *et al.* (T9) on the solubilities of inert gases.

^b The Bunsen's absorption coefficient is expressed in terms of cc of gas under standard conditions in cc of liquid.

^c The measure of radiation damage was based on a comparison of the minimum growth rate after irradiation compared with appropriate controls.

D10, D12) that rats exposed to multiple sublethal doses of irradiation on a fat-deficient diet have a lower average survival time than those on a similar diet containing cottonseed oil or methyl or ethyl linoleate. While cottonseed oil protects male rats of different ages, the fat protects only the old female rats against x-irradiation (C6). The suggestion was made that since the male animals had higher linoleate needs, they were depleted of EFA sooner on the fat-free diets than the females.

These findings have been confirmed by Ershoff (E8) who also showed that the addition of hydrogenated coconut oil to irradiated fat deficient mice produced lower survival than to mice on a fat deficient diet not supplemented with hydrogenated coconut oil. This observation checked the studies of Deuel *et al.* (D11) who found that the addition of hydrogenated coconut oil to an otherwise fat-free diet hastened the appearance of symptoms of essential fatty acid (EFA) deficiency in the rat.

The physiological effects of fatty acids are well known and insofar as they contribute to the general health of the organism and participate in cell metabolism they can be expected to modify radiation toxicity. Whether these effects would be good or bad cannot be predicted *a priori* because of the inadequacy of information. From the physiological standpoint, however, it is of interest to present the conjecture of Deuel (D12, p. 823) as

to the reasons for the beneficial effects of EFA against x-irradiation:

. . . Since there is some indication that linoleate is required for the growth of new tissues, as well as for the repair of damaged ones, the restitution of such injured tissues would proceed more rapidly in the cases in which an adequate supply of EFA was available . . .

It might be anticipated that fats could be protective by scavenging radiation produced peroxide circulating in the blood or present in "labile" states in tissues. It is also conceivable that fats might serve as a means of transporting peroxides or else of making peroxides available to intracellular sites. Consequently, it must be assumed that fats can function as protective or sensitizing agents, depending on the types of organic peroxides produced within the given fat; the location of the fat relative to structures such as the mitochondria and nucleus, the concentration of antioxidants naturally present in a given fat depot, and the rate of diffusion of peroxides into and out of the fat. If a fat protects by scavenging peroxides, it should possess some therapeutic effects when administered after irradiation.

The experiments of Ashikawa (A13) throw some light on the mechanism of lipid protection insofar as the mechanism is evaluated in terms of radiation chemistry. He found that olive oil was protective in the mouse but that the beneficial effect was small in the near total lethal dose of x-irradiation. However, in the mid-lethal range of radiation doses, survival of olive oil treated animals was about double the controls (75% to 43%). Other oils or fats such as peanut oil, methyl oleate, safflower oil, methyl stearate, and squalene were beneficial but that trilinolein, oleic acid, and methyl linoleate were actually harmful.

At first glance the protective action of olive oil which contains about 70 to 86 per cent oleic acid seems to contradict the fact that purified oleic acid was harmful. The reason appears to lie in the fact that olive oil contains antioxidants while purified oleic acid does not. Hence, the injection of pure oleic acid produces lipid hydroperoxides immediately upon entry into the animal and consequently, rather than act as a scavenger, it actually

adds to the peroxide levels. The injection of purified oils is mildly toxic which suggests, as Ashikawa (A13) points out, that a lack of antioxidant in the animal's tissues capable of coping with a sudden increase in oxidizable acid results in toxicity. In part, the toxicity of pure unsaturated fats can be reduced by addition of antioxidants directly (D12, A13). Similarly, safflower oil (51 to 79% linoleic acid) containing antioxidants is beneficial while methyl linoleate was harmful.

It might be expected that antioxidants which can become incorporated in body fats would have a protective effect. Ershoff and Steers (E9) have shown experimentally that mice exposed to multiple sublethal doses of total body x-rays and fed antioxidants in the diet could have their survival time significantly prolonged. Not all substances with antioxidant ability were effective. They found, for example, that butylated hydroxy toluene, propyl gallate, and 2,5-di-tert-butyl-hydroquinone were effective when fed at levels of 0.25 or 0.5 per cent of the diet but that mixed tocopherols and Santoquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline) were ineffective. The differences in activity could be due to capabilities of reaching sites of peroxide formation, metabolic stability, etc. Suitable antioxidants might be chosen on the basis of their known metabolic fate as given in the comprehensive review of Williams (W10).

The choice of specific antioxidants can be aided by the comprehensive description given by Deuel (D9, Chapter III). These include the phenols, quinones, tocopherols, gossypol, sesamol, dianilinogossypol, nordihydroguaiaretic acid, gum guaiac, galates, tannins, and many others. It is of interest to consider the use of synergists to bolster the effectiveness of antioxidants. Synergists include ascorbic acid, various amino acids, and proteins. An interesting classification of synergists to be used with antioxidants is available (D9, p. 299).

PHYSICAL CHEMICAL FACTORS

The absolute affinity of the copper sites in the copper oxidases for oxygen and the rate of oxidation of the cuprous copper state can be expected to be dependent on conventional physico-chemical factors such as temperature, ionic strength, hydration,

and dielectric constant. Obviously, these factors are more easily varied in suspensions of cells or bacteria. Modifications such as lowering of temperature or reduction of ionic strength would presumably enhance the interaction with Cu(I) interacting protective agents.

Overall, the effects of changes in the physical chemical environment on radiosensitivity are difficult to predict unless accompanied by measurements of a specific nature such as *in vivo* peroxide yields. For example, consider the effects on radiosensitivity of feeding deuterium oxide to animals. In one report (K2) the body water in mice was replaced progressively. From one standpoint, sensitization might be expected since radical and molecular yields are usually higher in heavy water. On the other hand, oxygen is less soluble in D₂O. Reaction rates including enzymatic processes are decreased. It was found, in fact, that a slight protective effect existed, the LD₅₀ increased progressively as the levels of D₂O in the body fluids increased. At very high D₂O levels, 30-35 atom per cent, however, a slight sensitization was noted presumably caused by the additive toxic effect of the high D₂O levels.

Generally, changes in the body water of mammals would not be expected to be reflected in the radiation yields within the lipid membranes of the mitochondrial structures.

Chapter XI

RADIATION SENSITIZATION

SENSITIZING AGENTS appear to act like additional radiation according to Mitchell (M25). This is what is also required by that part of the Cu(I,II)-Peroxy mechanism expressed in equations VI.9 to VI.14. Consequently all agents which preferentially form stable chelates with Cu(II) *and* can contact the intracellular copper in sufficient concentration are potential sensitizing agents. In addition, agents which are capable of forming non-dissociable chelates with either Cu(I) or Cu(II) are potential sensitizers. Any agent which increases intracellular peroxide formation either chemically or as a result of interaction with ionizing radiation is a potential sensitizer.

If the action of chemical sensitizers is fundamentally equivalent to ionizing radiation insofar as oxidation of mitochondrial copper is concerned, then chemical protective agents against ionizing radiation should also reduce the toxicity of the chemical radiosensitizers. Phillips and Cater (P7) showed that pre-injection of cysteine or glutathione protects rats from lethal doses of Synkavit (p. 170). Many sulfhydryl compounds effective as prophylactics against radiation toxicity have been found to protect against the lethal effects of ozone, and oxygen (B7, F1) and nitrogen oxide.

Apparent or feigned sensitization may result from the administration of chemically toxic levels of an agent because of the synergistic action of two insults. However, such synergism can often be distinguished from true sensitization by the fact that a high proportion of very early deaths is found in chemically poisoned animals receiving the usual acutely lethal doses of radiation.

Another subtle effect must be considered which could have

the effect of producing an apparent net protective action. At near toxic doses many chemicals produce hypoxia which would then tend to counteract the radiosensitizing action. Consequently, the design of experiments for evaluating sensitizing action must be carefully made. For example, reliable observations of radiosensitization with radiation doses producing an LD₉₉ in thirty days are practically impossible. It is necessary to operate in the region of the LD₅₀ and lower and employ much larger numbers of animals than are generally employed in evaluating protective agents at the LD₉₉ levels.

No systematic experimental studies of radiation sensitization have been made in the sense that important variables such as radiation dose, dose rate, and drug dosage were varied over a wide range. Consequently, one group of investigators may report that a given compound possesses sensitizing action while another group fails to find the reported sensitization. Yet both groups may be correct!

SENSITIZATION BY AMINO ACIDS

Alanine

A chelating agent showing preference for Cu(I) can be converted into a Cu(II) preferring agent. Consequently, this could mean that a protective action associated with the Cu(I) preference would be lost and changed into a sensitizing action because of the ability to form a stable Cu(II) chelate. An example of such an interesting phenomenon appears to have occurred in the series of studies by Langendorff and Koch (L3, K18). They apparently demonstrated that a significant degree of radiosensitizing action was imparted to mice administered a series of α -alkyl alanines. The authors were unable to explain this action, especially in view of the close structural similarity between the alanines and compounds of the cysteine-cysteamine group.

One explanation appears to lie in the fact that the alanines are Cu(II) preferring agents while the closely related sulfhydryl compounds are Cu(I) preferring reagents. Consider α -alanine and cysteine:

 α -Alanine

SENSITIZING AGENT

$$\text{Log } K_1 (\text{Cu}^{\text{II}}) = 8$$



Cysteine

PROTECTIVE AGENT

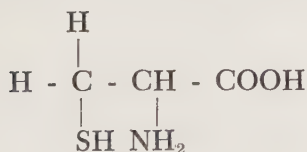
$$\text{Log } K_1 (\text{Cu}^{\text{I}}) = 19$$

It is interesting to note that α -l-alanine appeared to be a more effective sensitizing agent than the corresponding α -d-alanine. It is tempting to ascribe such a difference to a greater ability of the l form to contact enzymatic copper as may have been the case with radioprotective optical isomers of mercaptobutyl-2-guanidine (p. 139).

The α -alkyl alanines cannot sensitize by the mechanisms depicted by reactions VI.11-VI.13 because the stability constant is too small relative to the normally available amines in the cell. It is obviously impossible for compounds with $\log K_1(\text{Cu}) \sim 8$ to remove enzymatically bound copper where the $\log K_1(\text{Cu-protein}) \sim 18$ as is the case for the hemocyanin (F3) and the copper oxidases, hence for reactions depicted by equation VI.11 to occur are out of the question.

Cu (I) Chelating Agents

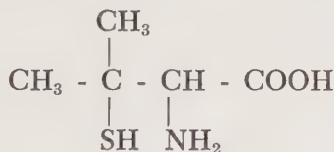
Consider the problem posed by the fact that cysteine is a protective agent while the closely related sulfhydryl compound penicillamine, in Langendorff's experiments at least (L4), produces sensitization. The structural formula and stability constants for copper of these compounds are shown below:



Cysteine

PROTECTIVE

$$\text{Log } K_1 (\text{Cu}^{\text{I}}) = 19$$



Penicillamine

SENSITIZING

$$\text{Log } K_1 (\text{Cu}^{\text{I}}) \cong 19$$

Langendorff's experiments were carried out with doses of x-rays

amounting to 500r which resulted in the survival of 28 per cent in the case of his control rats and 31 per cent of his control mice. The D-penicillamine treated rats received 240 mg/kg while the mice received 320 mg/kg and the resulting survivals were 8 and 12 per cent respectively—both results seem highly significant statistically.

Penicillamine is relatively resistant to metabolic degradation. In a systematic investigation of the enzymatic degradation of cysteine and derivatives, Aposhian (A11) found that substitutions on the β -carbon atom of cysteine increased the metabolic stability of the molecule. Penicillamine, the dimethyl substituted compound, proved to be far more resistant to degradation than cysteine. Further evidence for the remarkable stability of penicillamine relative to cysteine was shown in experiments in which it was found that penicillamine but not cysteine was able to protect animals acutely poisoned with copper or mercury salts. Finally, it has been demonstrated that the strong copper chelating agents ($\text{Log } K_1 \approx 15\text{--}20$) when administered for long periods leads to actual removal of copper from the copper protein ceruloplasmin, and to depigmentation and graying of hair in both animals and humans as a result of the *in vivo* removal of copper from the copper enzyme tyrosinase (L9).

Despite the metabolic stability of penicillamine, the presence of two methyl groups may prevent the molecule from making contact with the copper of cytochrome oxidase (pp. 50 and 138). This would appear indicated from the lack of inhibition of *liver* cytochrome oxidase observed following the injection of 100 mg/kg of D-penicillamine. However, this lack of effectiveness may be due to artifacts of assay (p. 87) as well or to too small a dose. In any event, we have not been able to observe either sensitization or protection by either the D- or L- forms of penicillamine and neither have other investigators, as for example, Doull *et al.* (D18).

We (S24) employed female CF-No. 1 mice whose ages varied from 8 to 9 weeks and in the case of one group treated with L-penicillamine, the mice were 18 weeks of age. They were injected intraperitoneally either pre- or post-irradiation and in some experiments multiple injections were given either

pre- or post-irradiation and sometimes in combinations of times. The mice received in most cases 750 r of x-irradiation which produced 100 per cent mortality after 30 days. The mean survival time was about 12 days for the 8-9 week old mice and 10 days for the 18 week old mice. A wide range of penicillamine doses was employed, namely 25, 50, 75, 100, 150, 175, and 250 mg/kg of the D-penicillamine and 1, 5, 25, 50, 75, 100, 125, and 150 mg/kg of the L-penicillamine. In no case was a significant degree of sensitization or protection observed, either from the standpoint of mean survival time or 30-day survival. Doull *et al.* (D18) found no affect on mean survival time or 30-day survival with CF₁ male mice, 6-8 weeks old, treated with doses of D-penicillamine in doses as high as 500 mg/kg prior to x-irradiation with 800 r.

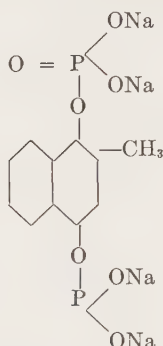
It would appear necessary that a purported sensitizing action be tested at different radiation doses so as to obtain a complete mortality curve for a given dose of a drug. The pitfalls in not doing so have been emphasized by Thomson (T7, Chapter 3). When the dose enhancement factor is low as is the case with penicillamine, it seems that data obtained with only a few points on the mortality curves are subject to variations, plus or minus, depending on many factors of a seemingly trivial nature.

SYNKAVIT

A sensitizing action by certain phenolic and quinoid derivatives can be expected in cases where the interaction cannot lead to reduction of Cu(II). Protection actions by phenols are known (see p. 149).

Consider the sensitizing action of the compound Synkavit studied by Mitchell and collaborators (M26), and having the structure shown on page 170.

Details of the metabolic fate of Synkavit are not known but it is probable that it can be hydroxylated by phenolase activity. The resulting compound containing ortho quinone groupings would have a pronounced affinity for Cu(II) with a $\log K_1 \approx 20$ and practically none for Cu(I). From an examination of the structures of the more than thirteen structural variations of Synkavit reported by Mitchell (M26), it seems highly significant that the inactive forms were generally those in which the location



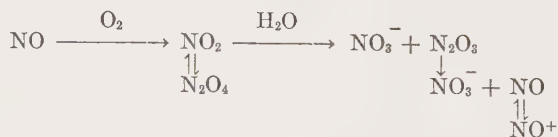
Synkavit (Tetra-sodium 2-methyl-1:4 naphthohydroquinone diphosphate)

of $-\text{CH}_3$ or $-\text{I}$ substituents would prevent the formation of ortho-diquinones—i.e., the formation of strong $\text{Cu}(\text{II})$ chelating agents.

NITRIC OXIDE

Nitric oxide, NO , appears to act, radiobiologically, in a way similar to oxygen toward human liver cells to x-rays, bacteria, bean roots, and Ehrlich ascites cells in “wet” systems. The protective action of nitric oxide in dry systems has been discussed earlier (p. 149).

Consider the chemistry of nitric oxide in a “wet” system. It readily combines with oxygen to form nitrogen peroxide, NO_2 . In water, NO_2 is first converted into nitric acid and N_2O_3 and then the latter changes into more nitric acid and nitric oxide which ionizes to the nitrosyl cation, NO^+ . The reaction sequence following, Sidgwick (S33), is summarized as follows:



The nitrosyl ion has the same structure as the neutral carbon monoxide molecule. Both form metallic complexes with many metals including copper but the nitrosyls differ from the carbonyls in one important respect insofar as copper is concerned—

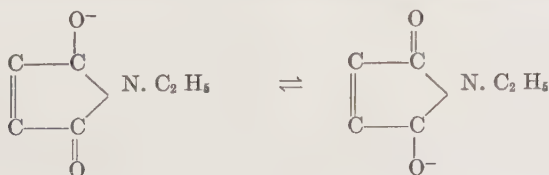
the nitrosyls unlike the carbonyls do not form cuprous complexes but do form nitrosyl complexes with cupric copper (S33).

The so-called sensitizing action of nitric oxide in wet systems as reported in the literature actually refers to oxygen-free systems in which the original radiosensitivity exhibited by an organism in the presence of oxygen is restored by the presence of nitric oxide. Whether a true sensitization or potentiation can take place, i.e., an increase in the radiosensitivity *above* that observed in the presence of oxygen does not appear to have been investigated. The "restoration" ability of nitric oxide in wet systems most probably involves the strong oxidizing action of nitrogen peroxide and other oxides produced when nitric oxide is dissolved in water.

THE HYDRATED ELECTRON

It has been suggested that the hydrated electron (p. 79) can produce radiobiological sensitization through the capture and stabilization of the electronic charge by the additive molecule (A1a). It is assumed that the negative radical-ion so produced, which damages a target molecule in the organism, is longer-lived than the free hydrated electron, thus extending its original sphere of activity. According to Adams and Dewey (A1a), a sensitizer is a molecule which possesses a high electron affinity together with a structure capable of resonance. The "increase in stability of the radical-ion relative to that of a non-resonating system could be responsible for increasing the likelihood of damage by prolonging the life-time of the electronic charge."

In general, molecules containing $>C=O$ linkages are very efficient electron scavengers. Such a molecule N-ethylmaleimide (NEM), has been found to possess marked sensitizing action toward *E coli B/r*. It was postulated that the hydrated electron acting on NEM produces the following resonating system:



Guided by the above model, the authors (A1a) tested benzophenone and diacetyl—the latter as an example of a pure non-aromatic non-cyclic conjugated di-ene system. These compounds as well as NEM gave dose reduction factors for *Serratia marcescens* irradiated in nitrogen. Elimination of the conjugated linkages in NEM in a molecule containing $> \text{C}=\text{O}$ groups was predicted to remove the sensitizing ability, as was observed in the case of succinimide.

It is well-known that NEM reacts with SH groups and this may be a factor in its radiobiological action as has been cited (A1a). It is also possible that the stabilization of the negative radical-ion may permit a more effective interaction with the copper of cytochrome oxidase. Consideration of the model by Anbar (A9a) discussed in Chapter V may be of interest in this regard as well.

CALORIC INTAKE

A few comments are given here regarding the effects of irradiation on animals which have been deliberately under-fed in terms of calories but given a diet adequate in all essential nutrients. It is known that calorie-restricted animals have a longer life span (C2, C12). They also have a decreased incidence of tumors.

Carroll and Brauer (C2) have found that the hypocalorically reared rat shows a highly significant reduction in its tolerance to radiation when compared to freely fed controls of the same age. No apparent sizable differences were observed with regard to pattern of mortality between the hypocalorically reared rats and rats fed *ad libitum*. They conclude that the decreased radiation resistance of the hypocalorically reared rats does not appear to be attributable to the size of the rats or to the stretching out of the life span.

The enhanced sensitivity of fasted rats can be interpreted in part as being due to increased permeability of mitochondria. It is known (D7) that in animals on a low calorie diet the mitochondria tend to swell. Consequently, they are expected to be more permeable to radiation-produced peroxides. Aside from the possible enhanced susceptibility of mitochondrial enzymes to damage, a lower lipid intake may also be involved.

Chapter XII

CHEMICAL TREATMENT POSTIRRADIATION AND AGAINST NEUTRONS

TWO AREAS OF RADIOBIOLOGY conspicuously unsuccessful are the chemical treatment of injuries suffered after irradiation and the protection against neutron irradiation. Actually, relatively little research involving chemical treatment has been conducted in these areas—presumably because of the lack of positive results. It is the purpose of this chapter to report on some of the more promising drugs and to outline how the Cu(I,II)-Peroxy mechanism leads to an experimental approach to these problems.

POSTIRRADIATION THERAPY

The development of biological treatment for injuries suffered after the delivery of irradiation has been the subject of considerable investigation (A13, B7, T7). The most promising approach has been the use of bone-marrow and spleen transplantation and other means of inducing cell proliferation and replacement. Chemical treatment has not been very successful, probably because of the fact that nuclear damage following lethal doses of irradiation probably occurs nearly instantaneously. However, in view of the contribution of cytoplasmic components to nuclear damage and vice versa it would appear that the scavenging of peroxy radicals or reduction in peroxide production would permit a chemical approach to postirradiation therapy, especially with doses of irradiation in the LD₅₀ range. This approach becomes especially promising in view of the delay in the production of peroxides in many tissues.

Theoretically, there are two approaches to chemical treatment which appear most logical. These are 1) the use of agents which directly or indirectly destroy peroxy radicals and 2) the

use of drugs which may react with enzymatic copper sites in the presence of peroxy radicals. In the first group are included the lipids and amines and in the second group are included the cyanides, nitriles and carbon monoxide.

Substances which mobilize calcium from the bone have been found to increase the 30-day survival percentage of rats even when injected after irradiation (R6). Two effective agents are parathyroid extract (PTE) and the sodium salt of ethylenediamine tetraacetic acid (Na_2EDTA). The suggested mechanism of therapeutic action against radiation injury has been described (p. 98). Rixon and Whitfield (R6) have reviewed other evidence implicating calcium and chromosomal damage relationships. Of particular interest insofar as permeability is concerned is the observation that calcium gluconate increases the survival of irradiated conidia of *Neurospora crassa* and at the same time prevents the radiation-induced leakage of phosphorus into the medium.

The effectiveness of PTE depends on dosage, not only of the hormone itself, but on the radiation dose, and the time of administration. However, above a dosage of 50 units of PTE, the survival rate reached a maximum and became independent of the dose of the extract. A significant degree of survival of x-irradiated rats was found at doses of 760r and higher, but curiously not with lower doses. With 200 units of extract the therapeutic action was at a maximum whether injected 5 minutes or 1 hour after irradiation but decreased to about half the maximum after 3 hours, and was ineffective when administered 5 hours after irradiation.

Best results were observed when 200 units of PTE were administered 5 minutes after irradiation with 800 r (Controls—33% survival, treated—73% survival). The efficacy of PTE decreased with lower or higher doses of irradiation. Following 700 r or 1,000 r no significant therapeutic action was found.

Injection of a non-toxic dose of Na_2EDTA (300 mg/kg) to rats immediately before or after irradiation with 740 r increased 30-day survival from 33% to about 70% (R6). The injection of the calcium salt of EDTA immediately before irradiation only slightly increased the survival rate above the controls. Higher doses of

Na₂EDTA acted synergistically with radiation resulting in an increased proportion of "intestinal" deaths within the first week, though the 30-day survival percentage was higher overall. It was suggested (R6) that Na₂EDTA sensitized the intestinal tissue to x-irradiation by the removal of calcium and possibly magnesium.

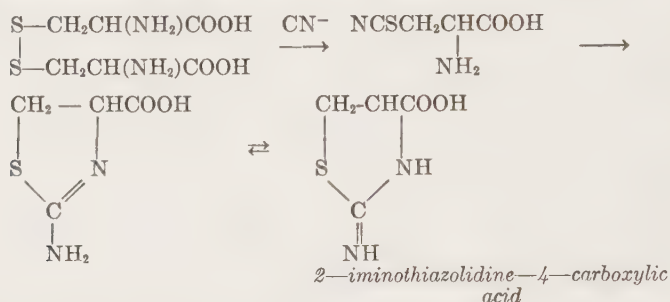
Postirradiation treatment with olive oil has been beneficial (A13). These studies indicated that the postirradiation oil treatment does not reverse radiation damage but facilitates the recovery process. The olive-oil treated animals were twice as fertile as the untreated groups, but by 60 to 75 days postirradiation both groups appeared completely recovered. The chemical nature of the therapy was demonstrated in many ways. The possibility that physical factors such as an inflammatory reaction stimulating the reticulo-endothelial system or an emollient action of the oil on the intestines was ruled out—an important point because some protection is obtained with ground glass or powdered limestone.

Small postirradiation effect in terms of the rate of death of mice administered the calcium-sodium salt of EDTA has been observed (R6, S24). In these cases it is assumed that the EDTA is acting as a tertiary amine and thus destroys peroxides (p. 151).

Cyanides, CO, and nitriles are interesting for postirradiation protection because they can protect the copper oxidase even in the presence of peroxides. In early studies (B6) Bacq and co-workers found that cyanide delayed the onset of death in mice given a lethal dose of radiation compared to the controls, even though the 30-day survival times showed no differences.

Inhalation of 0.10% carbon monoxide by male guinea pigs after receiving an LD_{50/30} dose of x-irradiation (250r) resulted in "up to 85% survivors" (K22). In guinea pigs receiving 500r the 30-day survival for animals breathing 0.10% CO for three days post-irradiation was 15% compared to no survival in the controls and 50% in the pre-treated animals. Rabbits irradiated with 1000 r were not protected by post-CO administration. The slight and limited though statistically significant ($P < 0.05$) result on the guinea pigs treated post-irradiation with CO is, according to the investigators (K22), apparently not due to hypoxia since hypoxia *per se* is not effective after irradiation.

Some of the most promising results of chemical treatment post-irradiation have been obtained by Dilley and Doull (D13a). They observed that cyanide and one of its metabolites, 2-iminothiazolidine-4-carboxylic acid (ITCA) (p. 124) had therapeutic and prophylactic effects against radiation lethality in mice. The ITCA can be prepared by mixing cystine and cyanide. The suggested mechanism of the reaction is as follows (W10, p. 392):



It has been shown, using ^{35}S -L-cystine that ITCA was derived *in vivo* from cystine.

Another compound tested by Dilley and Doull was sodium sulfite inasmuch as its reactions with disulfide groups are similar to cyanide but slower (see reaction equations for *copper-cysteine-sulfite* in Chapter V.)

In their studies (D13a) female CF_1 mice weighing 20-25 grams were used. They were irradiated with 600 r of x-rays with a dose rate of 235 r per minute. Thirty minutes after the irradiation—a previously determined time for maximum therapeutic effect—the mice were injected intraperitoneally with different drugs. At the end of 30 days the survival of the controls was 20% compared to the following treated groups: sodium sulfite-300 mg/kg, (11/32 = 34%); ITCA-750 mg/kg, (8/16 = 50%). No therapeutic effect was observed when 500 mg/kg of cystine was mixed with 300 mg/kg of sodium sulfite or when a lower dose of ITCA, 500 mg/kg, was administered.

The mechanism of action of sodium sulfite and cyanide was discussed (D13a) in terms of their inhibitory effect on enzymes including the cytochromes. They pointed out that the inhibition could be reversed if cystine were given at the same time or im-

mediately afterward. With reference to the therapeutic action of sulfite they commented:

"The fact that in these series of experiments we were able to abolish the protective effects of sodium sulfite by giving cystine at the same time suggests that these particular enzyme systems may be target molecules for damage produced by irradiation. This effect is probably not seen with cyanide since it reacts directly with cystine very quickly, even at room temperature. While sulfite ion undergoes an analogous reaction it takes place much slower."

Aside from or in combination with the ability of $\text{SO}_3^{=}$ and cyanide to reduce disulfide groups, one could postulate that they reactivated cytochrome oxidase by reducing Cu^{++} to Cu^+ . On the other hand, an explanation for the therapeutic action of ITCA seems more obscure. It is stated that ITCA is inert metabolically and is excreted unchanged by rats when fed or injected (W10, p. 392). However, it is probable that part of the ITCA does react. It is known that thiazolidine-4-carboxylic acid is broken by liver mitochondria with the formation of N-formylcysteine (W10, p. 618). Though it should be pointed out that thiazolidine-4-carboxylic acid in doses of 50 to 100 mg/kg lacks radioprotective action (D18, p. 83) higher doses corresponding to those of ITCA might prove effective. However, this is precluded by the relatively high toxicity of thiazolidine-4-carboxylic acid ($\text{LD}_{50} = 100\text{-}200$ mg/kg) compared to ITCA (>750 mg/kg).

It is reasonable to assume that a combination of a cyanide or nitrile with an amine or oil might be more effective than either compound alone. Such a combination would serve the purpose of reducing the peroxide levels, especially in view of the possibility that cyanide alone may result in higher than normal levels of peroxides following irradiation. In preliminary experiments using a combination of malononitrile and EDTA promising results were obtained (S24). Mice given the combination following irradiation with an LD_{90} dose of x-rays were still alive 15 days after irradiation while 90 per cent of the controls were dead. However, the treated animals subsequently died so that the 30-

day survival times were about the same as the controls. The data indicated the delay in death amounted to an additive effect of the nitrile and EDTA. More promising combinations for *experimental animals* would include cyanide or cysteamine and Na_2EDTA since cyanide or cysteamine do not react with calcium. Not excluded is the addition of antibiotics.

PROTECTION AGAINST NEUTRON IRRADIATION

Pretreatment with cysteine confers significant protection against the lethal effects of gamma rays and fast neutrons (P3). However, the effectiveness against the fast neutrons is only about a third to a half as effective as against gamma radiation. In other studies Vogel *et al.* (V3) showed that the administration of AET or serotonin to mice before irradiation with gamma radiation gave effective protection but that these agents were far less effective against an acutely lethal dose of fast neutrons. It has also been stated that anoxia does not protect against neutron exposure (D20).

It is well established that the effects of neutron, alpha particle, and other heavy ionizing radiation are less sensitive to the presence of oxygen than x- or gamma irradiation (B7). In growing roots it has been demonstrated that the radiosensitivity against x- and gamma irradiation can be reduced by anoxic conditions (D4). In this case the sensitivity refers to the frequency of chromosome breaks. In the case of chromosome breaks induced by α -particles and neutrons, the effect of changes in oxygen tension is reported to be slight (D4, p. 177).

Aside from possible direct actions, it is here postulated that protection against neutron irradiation can best be accomplished by those drugs which act by the same mechanism as cyanide, in other words, agents which act by direct protection of an intracellular radiosensitive site, e.g., the cytochrome oxidase in mitochondria. In these cases, the site would be unaffected by neutrons despite a more efficient production of peroxy radicals by neutrons. It is further conceivable that the protection afforded by drugs against neutron irradiation is due to that part of the action involving direct interaction with the aforementioned intracellular sites. Consequently, it could be surmised that a third

to a half of the radioprotective action of cysteine is due to the same mechanism of action as cyanide (see p. 131 for discussion involving bacteria). It is predicted that cyanide and nitriles should be about as effective against neutron irradiation as against x-rays and gamma rays. In fact, hydroxycyacetoneitrile has been found to be the most successful protective agent in mice against fast neutrons (T7).

Other possible drugs are those involving a mechanism of action by which peroxy radicals are destroyed. In other words, the same types of agents which would be effective when administered postirradiation should offer the best possibilities for protection against neutron irradiation.

Chapter XIII

COMPARATIVE RADIOSENSITIVITY OF ANIMAL ORGANISMS AND TISSUES

The doses of ionizing radiation which kill animal organisms vary by a factor of roughly a thousand (B7). An explanation for this wide range of radiosensitivity has been suggested by Sparrow and Evans (B26a, pp. 76-100) and Puck (P17). Their studies indicate that to a first approximation the dose of radiation necessary to produce reproductive death is an inverse function of the DNA content per diploid nucleus or the interphase nuclear volume. In their work with plants Sparrow and Evans found that the daily dose rate of radiation required to produce severe growth inhibition in six different species of plants was correlated with the average amount of DNA per nucleus even though the DNA values per nucleus varied about three hundred-fold. They applied the same relationships to some animal organisms and microorganisms by making estimates of their DNA content and found that the radiation dose presumably required to inhibit cell division in plants and animals of similar DNA contents were in reasonable agreement.

Puck (P17) reported a suggestive correlation between the LD_{50} for thirty days for a few multicellular organisms and the dose, D_0 , in roentgens necessary to reduce the reproductive fraction of the cell population to 37%. He compared the LD_{50} for three whole warm-blooded organisms—man, Chinese hamster, fowl—and found that the D_0 values for their single cells measured *in vitro* varied in the same way. However, since the D_0 values varied by a factor of eight compared to three for the LD_{50} values, these few data possess only provisional significance. More impressive correlations between DNA content and D_0 for different species have been reported by Puck.

The Cu(I,II)-Peroxy mechanism suggests that there should exist a correlation between the radiosensitivity of aerobic multicellular organisms to acutely lethal doses of radiation and their copper contents. The existing experimental data show that there indeed seems to be such a correlation which permits the LD₅₀ to be estimated on both a relative and absolute basis. This could imply that the components of radiosensitivity associated purely with the nuclear or chromosomal structures independent of the contributions of the cytoplasmic components are either roughly constant or relatively small compared to those factors which contribute to the LD₅₀ of different species. In this connection it is instructive to note that differences in the genetic sensitivities of different species appear to be far less than the corresponding differences in the LD₅₀. For example, the induced rate of mutations at seven loci studied in the mouse is about fifteen times that for a comparable group of loci in *Drosophila* (R4, p. 95). According to Conger (H12, p. 216) induced mutation rates in the mouse are about three to eight times higher than in *Drosophila*. On the other hand, the dose of x-rays or γ -rays needed to kill insects including *Drosophila* is about one hundred times greater than that needed to kill mammals including the mouse (B7, p. 304).

A discussion of comparative radiosensitivities of animal species involves information of the complex interactions of numerous factors and time relations, some known and many unknown. On the basis of current knowledge it would be presumptuous to attribute a phenomenon as intricate as the LD₅₀ of multicellular organisms to a single mechanism such as, for example, the production of chromosome breaks by direct "hits" of ionizing radiation. One could assume, however, that all lethal effects stem from damage to the nuclear components from both direct and indirect actions of radiation within and without both the nucleus and cytoplasm. Be that as it may, it is worth examining the extent, even empirically, to which copper levels correlate LD₅₀'s of different organisms.

In reality, it is preferable to distinguish between biochemically active copper and extraneous inactive copper whose concentrations depend on the vagaries of diet. It will be assumed

as a first approximation that the total body copper of an organism is proportional to the "biochemically active" copper and that this proportion is roughly the same for all species. In this connection it is worth noting that the percentage of copper in the mitochondrion of human liver cells is about equal to that of the rat. In the case of two specimens of human liver tissue, it was found that mitochondrial copper amounted to 7.4 and 9.4 per cent, respectively of the total copper (E4). Information on the subcellular distribution of copper and other trace metals has been given in Table III.2.

It is recognized that even though two species may contain the same total copper content, one species might prove more radiosensitive because of an especial sensitivity of a critical organ or unusual levels of antioxidants or peroxides. It is, of course, preferable to obtain the relative amounts of copper in the mitochondria and other cell particulates of individual organs, since these may vary considerably from species to species, even though overall copper contents are equal. Further, since much of the mitochondrial copper is associated with cytochrome oxidase, it would be expected that species radiosensitivity would be a function of relative cytochrome oxidase levels and mitochondrial number as well. However, in some organisms, especially unicellular ones, the cytochrome oxidase levels are very low and it is probable that the cytochrome system does not function as in aerobic multicellular organisms (p. 189).

In view of the nearly complete lack of any explanation available for differences in the observed radiosensitivity of different species, no apology is offered for the introduction of certain speculations. Rather, the worthwhileness of the concepts presented here will be their ability to correlate or to anticipate experimental observations. It is hoped that they will lead to a systematic and extensive series of measurements of mitochondrial copper levels and cytochrome oxidase levels in the organs of different species. Further, it would be worthwhile to ascertain whether the radiosensitivity of an organism can be increased by rendering them copper deficient.

In view of the large differences in radiosensitivity associated with variations in copper content, it would be of particular in-

terest to apply these findings to the treatment of cancer by irradiation. For example, the copper levels in a tissue such as the liver can be reduced by repeated administration of penicillamine (p. 168) prior to irradiation of the growths. If the copper levels in the tumor are reduced at a greater rate than the normal tissue an impressive improvement of irradiation treatment might ensue.

RADIOSENSITIVITY AND COPPER LEVELS

Animal Organisms

Assuming a proportionality between total body (Cu_t) copper and the LD_{50} —30 days, it is possible to calculate the LD_{50} for a species from the known value of the LD_{50} and Cu_t for a "reference" organism. Taking the rat as the reference species, the LD_{50} of "Species X" would be given, roughly, by the expression:

$$\frac{[LD_{50}(\text{Species X})]}{[LD_{50}(\text{Rat})]} = \frac{[(Cu_t)(\text{Species X})]}{[(Cu_t)(\text{Rat})]} \quad (\text{XIII.1})$$

From existing data (B7), the LD_{50} of the rat is taken as 800r while Cu_t is taken as 8 mg/kg on a *dry* weight basis. Substituting into equation XIII.1 we obtain for any animal organism that:

$$LD_{50} \cong 100 (Cu_t) \quad (\text{XIII.2})$$

The estimated LD_{50} for different organisms is given in Table XIII.1

The correlations in Table XIII.1 are remarkably good and

TABLE XIII.1
RADIOSENSITIVITY OF DIFFERENT SPECIES AS A FUNCTION
OF TOTAL BODY COPPER CONTENT^a

Organism	Mg/kg dry wt.	Calc. LD_{50}	Range of Observed LD_{50}
Mammals	2-12	200-1200	200-1200
Insects	92	10,000	$10^3 - 10^5$
Marine invertebrates	170	20,000	$10^3 - 10^5$

^a The over-all average copper contents for the three classes of organisms were obtained from Dethier (D8). The estimated ranges in copper content are from the extensive tabulations of Brenner (B25).

may be improved when the range of copper levels for individual organisms are considered. In the case of man (C3) the copper concentration is about 7 mg/kg (5.5 — 8.5) of dry weight which would yield 550 — 850r as the estimated LD₅₀. For the following animals: mouse, rat, dog, rabbit, guinea pig, sheep, horse, and fish, the overall copper content covers a six-fold range (B25) corresponding to the observed range of the LD₅₀.

An absolute estimate of the LD₅₀ based on the concentration (E4, T5) of mitochondrial copper levels, (Cu_m) can be made. In man, for example, the copper concentration amounts to approximately 1.7 mg of copper per kg of *fresh* weight or, assuming that 8% is "active", roughly 0.14 mg or 2 micromoles of (Cu_m) per kilogram. Assume that the radiation yield, G_o(Cu⁺⁺) = 2 as determined experimentally *in vitro* (S23) and *in vivo* (S24) for the copper protein, hemocyanin, for the horseshoe crab, *Limulus polyphemus*. On this admittedly speculative basis, the maximum radiation dose necessary to completely destroy the ability of (Cu_m⁺) in man to react with molecular oxygen would be 1,000r.

In the horseshoe crab the principal oxygen carrier in the blood is hemocyanin. This species, *Phylum arthropoda*, is presumably highly resistant to radiation (B7). The concentration of copper in the blood of *Limulus* is about 10⁻³ M. From the G(Cu⁺⁺) value, it is calculated that the amount of x-irradiation necessary to destroy 50 per cent of the oxygen-carrying capacity of the crab would require about 250,000 rads. This is probably a maximum value because it does not include the concomitant damage to cell nuclei and other copper oxidases.

Tissues

From the known copper contents of normal adult human tissues tabulated in Table III.1, a correlation between copper content of a tissue and radiosensitivity is suggested. Thus, the organs with the highest copper contents are the liver, brain, and heart, while the spleen, and especially the leukocytes are among the lowest. However, until accurate values of the mitochondrial copper levels are available for all organs it is not worthwhile to make further comparisons on this basis.

The total number of mitochondrial copper atoms in a cell

provides a basis for estimating the number of peroxy radicals needed to inactivate the total cytochrome oxidase present and for estimates of the number of large ionizing particles such as alphas and neutrons which might be required to inactivate all the cytochrome oxidase in a given cell by direct hits.

Sufficient data are available on rat liver to estimate the total copper atoms present in the mitochondria. Allard *et al.* (A5) have reported that there are 1.3×10^8 liver cells per gram fresh rat liver and a total of 33×10^{10} mitochondria per gram fresh liver. Hence the average number of mitochondria per liver cell is 2.5×10^3 . Actually, other investigators have reported values ranging from 500 to 2,500 mitochondria per rat liver cell. The concentration of copper in rat liver fractions (young adult males, Hisaw strain) has been determined (T5). In one gram fresh liver 4.7 mg of copper were present and the mitochondria contained 8.2 per cent of the total liver copper.

From the information in the preceding paragraph the total number of copper atoms present in the mitochondria from one gram of fresh liver is 2.5×10^{10} , and an individual mitochondrion contains 10^7 atoms of copper. Considering the range of copper present in different cells, it would appear that roughly 10^8 to 10^{10} heavy particles would be needed as a minimum to inactivate all the mitochondrial copper in a cell if direct hits were involved or a minimum of 10^8 to 10^{10} peroxy radicals. It is interesting to speculate whether these numbers of copper atoms bear a simple relationship to the numbers (10^6 — 10^8) of heavy ionizing particles found to be lethal to cells in which only the cytoplasm has been irradiated (M25).

RADIOSENSITIVITY AND CYTOCHROME OXIDASE LEVELS

A suggestive correlation between cytochrome oxidase levels, radiosensitivity, and the age of animals has been discussed in Chapter X. A fundamental basis for examining a possible basis for correlating comparative radiosensitivity of tissues and cells would appear to be the levels of cytochrome oxidase (Table XIII.2). Again, a reasonable correlation exists for the relative radiosensitivities of different tissues as normally known (P4) and their cytochrome oxidase levels. Apparent exceptions appear to be

TABLE XIII.2
RELATIVE CYTOCHROME OXIDASE LEVELS
IN NORMAL HUMAN TISSUES^a

<i>Tissue</i>	<i>Relative Cytochrome Oxidase Activity (Cardiac Muscle = 1)</i>
Cardiac muscle	1.0
Brain	0.5
Kidney	0.4
Liver	0.3
Skeletal muscle	0.3
Adrenal	0.09
Thyroid	0.07
Intestine	0.06
Stomach	0.06
Pancreas	0.06
Spleen	0.05
Lung	0.05

^a After Greenstein (G9, p. 444).

mainly due to tissues composed of cells with very high nuclear-cytoplasmic ratios. A serious complicating factor is the lack of data on the concentration of cytochrome oxidase within the different regions of a tissue or specific cell types comprising the tissue.

Insufficient data exist with which to make other than a superficial attempt to compare cytochrome oxidase levels in the tissues of different species with their interspecies radiosensitivities. The paucity of data is especially bad when it is considered that the tabulated radiosensitivities of different strains of different species also varies, so that unless the enzyme levels were measured for the identical strain at comparable ages and conditions, adequate comparisons are not possible. It is of interest, however, to record some known measurements of the cytochrome oxidase levels of different tissues of various species along with their known ranges of radiosensitivity with the hope that systematic and controlled studies will become available in the future. The comparative cytochrome oxidase values and radiation sensitivities are tabulated in Table XIII.3 from data available in the literature.

RADIOSENSITIVITY AND MITOCHONDRIAL NUMBER

It would appear that mitochondrial number might provide a simple cytological index of relative radiosensitivity. However,

mitochondrial number is not necessarily an index of the oxidative metabolism so that care must be taken. In this connection the experiments of Miller *et al.* (M23) are pertinent. They determined the number of mitochondria per cell by different methods and the qO_2 of tissue and mitochondrial fractions for different tissues. The numbers of mitochondria are in the order brain > liver > kidney. This order corresponds to the usual accepted notions of relative tissue radiosensitivity. However, the relative differences in mitochondrial number are somewhat compensated by the metabolic activity of the individual mitochondrion which, in terms of qO_2 , is inversely proportional to mitochondrial count.

In the case of liver, about 30 per cent of the nuclei are of the small type usually believed to represent nonparenchymal cells, and in the case of brain about 60 per cent of the nuclei are quite small and probably belong to glial cells. Hence, the "average" mitochondrial count per cell is an abstraction in the case of these tissues, since little is known about the relative parenchymal and nonparenchymal cells (M23). However, when the same tissue under different experimental conditions is compared, these numbers are useful.

In a given cell the number of mitochondria appear to remain roughly constant (N4). In protozoa an enormous number of mitochondria may be found, e.g., up to 500,000 mitochondria in the giant ameba *Chaos chaos*. The egg of the sea urchin, *Psam-*

TABLE XIII.3

COMPARATIVE CYTOCHROME OXIDASE ACTIVITY OF THE NORMAL TISSUES OF VARIOUS SPECIES AND CORRESPONDING LETHAL RADIATION OF X-RAYS (LD_{50} —30 DAYS)

Species	LD_{50} (rem) ^a	Tissue Cytochrome Oxidase Activity ^b					
		Heart	Kidney	Brain	Liver	Skeletal Muscle	Spleen
Man	600-700	8.2	3.6	3.9	2.8	2.4	0.4
Rat ^c	590-970	40	14	13	12	6.3	4.3
Mouse ^c	550-665	19	11	9.6	8.0	2.8	2.0
Rabbit	750-825	9.5	4.7	4.9	2.9	2.0	0.8
Guinea pig	175-400	9.6	5.4	6.0	4.2	1.6	0.9

^a The LD_{50} data are taken from ref. B7, p. 300.

^b The cytochrome oxidase data are from ref. G9, p. 405, and are expressed in terms of cubic millimeters oxygen consumed per milligram wet weight per hour.

^c The cytochrome oxidase values for the rat were determined on tissues from Buffalo rats while the mouse tissues were from Strains A and C3H mice.

mechinus miliaris, contains about 150,000 mitochondria, while two other sea urchins are reported to possess 14,000 and 33,000 each.

In general, it appears that for cells of the same type, that mitochondrial number can provide a rough index of radiosensitivity. However, in the case of cells of different age, this is not necessarily true because of differences in cytochrome oxidase activity per mitochondrion. See page 158 for a discussion of mitochondrial number and radiosensitivity in relation to embryonic and neoplastic tissues.

RADIOSENSITIVITY AND CYANIDE RESISTANCE

Cytochrome oxidase is markedly sensitive to cyanide *in vivo*. The degree of inhibition is proportional to copper content inasmuch as the activity of the cytochrome oxidase molecule is directly governed by the copper component (p. 49). Consequently the response of an aerobic organism to cyanide could presumably be a measure of mitochondrial copper levels or cytochrome oxidase assuming that the cytochrome system is the principal one involved in respiration.

The relationship between cyanide resistance (in the pharmacological sense) and radiosensitivity could be more clearly discerned in organisms present in a suspension which do not possess a system such as hemoglobin which would divert the cyanide from interactions with the copper of cytochrome oxidase.

A parallelism between radiosensitivity and cyanide resistance has been reported by Bacq *et al.* (B7, B24), in different species of ciliated Infusoria. The results are summarized in Table XIII.4 where it is demonstrated that the higher the apparent resistance to radiation the higher is the resistance to cyanide.

Other examples (B7, p. 304) of a relation between cyanide resistance and radiosensitivity are found with the Coelenterates, except the hydrae. The Coelenterates can tolerate 0.4 per cent cyanide while the hydrae which are much more sensitive to cyanides are also much more sensitive to radiation. It is of interest to note that the mammalian retina is comparatively cyanide and radiation resistant.

TABLE XIII.4
PARALLELISM BETWEEN CYANIDE TOXICITY AND RADIOSENSITIVITY
IN OPHRYOGLENIDAE (THERONITES)^a

<i>Species</i>	<i>Ophryoglenida pectans</i>	<i>Deltopylum rhabdoides</i>	<i>Ophryoglenida atra</i>	<i>Ophryoglenida mucifera</i>
Dose of x-rays lethal in 10-15 minutes (kr)	600	750	900	1050
Max. well tolerated con'cn of NaCN	0.03%	0.05%	0.05%	0.2%

^a Data are taken from Bacq *et al.* (B4, B7).

Unicellular organisms are generally very resistant to ionizing radiations (B7, p. 300). The respiration of many unicellular organisms are, in fact, affected little or not at all by cyanide. According to Baldwin (B9, p. 174) it is probable that in cyanide resistant organisms the cytochrome system is not normally concerned in the respiration, or else that carrier substances of some other kind are present.

In studies on cyanide resistance and radiosensitivity it would be desirable to investigate quantitatively factors such as the degree to which cyanide inhibited cytochrome oxidase and the concentrations of peroxide produced by radiation.

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